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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 31/00, 39/00, C12N 15/00

A1

(11) International Publication Number: WO 96/10400

(43) International Publication Date: 11 April 1996 (11.04.96)

(21) International Application Number: PCT/US95/12507

(22) International Filing Date: 29 September 1995 (29.09.95)

(30) Priority Data:

| | | |
|---|---|---|
| 08/316,438 | 30 September 1994 (30.09.94) | US |
| 08/245,587 | 7 June 1995 (07.06.95) | US |


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(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

**Published**
*With international search report.*

(54) Title: GENE THERAPY VECTORS AND VACCINES BASED ON NON-SEGMENTED NEGATIVES STRANDED RNA VIRUSES

(57) Abstract

Recombinant methods for recovering wildtype or engineered negative stranded, non-segmented RNA virus genomes containing non-coding 3' and 5' regions (e.g. leader or trailer regions) surrounding one, several or all of the genes of the virus or one or more heterologous gene(s) in the form of ribonucleocapsids containing N, P and L proteins, which are capable of replicating and assembling with the remaining structural proteins to bud and form virions, or which are only capable of infecting one cell, or are transcribing particles, are disclosed. Novel vaccines, gene therapy vectors and antiviral compounds based on these viral particles are also disclosed.

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# GENE THERAPY VECTORS AND VACCINES BASED ON NON-SEGMENTED NEGATIVE STRANDED RNA VIRUSES

## Background of the Invention

Virus families containing enveloped, single-stranded, negative sense (3' to 5') RNA are classified into groups having non-segmented genomes (i.e. order Mononegavirales, which includes the Paramyxoviridae and Rhabdoviridae families) or those having segmented genomes (Orthomyxoviridae, Bunyaviridae and Arenaviridae families).

Of the non-segmented viruses, the Rhabdovirus family is perhaps the most common. Rhabdoviruses cause disease and infect vertebrate and invertebrate animals and plants. For example, the rhabdoviruses that cause rabies and economically important diseases of fish appear to have life cycles confined to vertebrate species. However, all other rhabdoviruses are thought to be transmitted to vertebrates and plants by infected anthropods, which may be the original hosts from which all rhabdoviruses evolved. Characteristically, all rhabdoviruses have a wide host range, although many have been adapted to grow in specific hosts and particularly at the ambient temperature of their hosts.

The viruses of the family Rhabdoviridae known to infect mammals, including humans, have been classified into two genera: the *Vesiculovirus* genus stemming from vesicular stomatitis virus (VSV) and the *Lyssavirus* genus otherwise known as the rabies and rabies-like viruses. The well-characterized viruses of these two genera include: 1) *Genus Vesiculovirus* - VSV-New Jersey, VSV-Indiana, VSV-Alagoas, Cocal, Jurona, Carajas, Maraba, Piry, Calchaqui, Yug Bogdanovac, Isfahan, Chandi pura, Perinet, Porton-S; and 2) *Genus Lyssavirus* - Rabies, Lagos bat, Mokola, Duvenhage, Obodhiang and Ko fon Kan.

VSV has a non-segmented negative-stranded RNA genome of 11, 161 nucleotides that encode five viral proteins: The nucleocapsid protein (N), the phosphoprotein (P, also called NS), the matrix protein (M), the glycoprotein (G) and the RNA-dependent RNA polymerase (L).

The Paramyxovirus family includes the morbilliviruses (e.g., human measles virus, canine distemper virus, rinderpest virus of cattle), the paramyxoviruses (e.g. sendai virus; human para-influenza virus types 1-4; mumps virus; simian virus type 5; and newcastle disease virus) and the pneumoviruses (e.g., human and bovine respiratory syncytial viruses (RSV), pneumovirus of mice and turkey rhinotracheitis virus) genuses.

The pneumovirus human respiratory syncytial virus (hRSV) is the major viral cause of serious lower respiratory tract disease (e.g. bronchiolitis and pneumonia) in infants and children. Similarly, bovine respiratory syncytial virus (bRSV) causes respiratory disease in cattle.

RSV have been isolated from a number of mammals including chimpanzee (Morris, J.A., et al., (1956) *Proc. Soc. Exp. Biol. Med.*, 92, 544-549); humans (Lewis, F.A., et al., (1961) *Med. J. Aust.*, 48, 932-933); cattle (Paccaud, M.F. and C. Jacquier, (1970) *Arch. Gesamte Virusforsch*, 30, 327-342); sheep (Evermann, J.F., et al., (1985) *Am. J. Vet. Res.*, 46, 947-951); and goats (Lehmkuhl, H.D., et al., (1980) *Arch. Virol.*, 65, 269-276). Human RSV (hRSV) have been classified into two subgroups A and B, which include a number of strains (e.g. A2 and 18537). A number of strains of bovine RSV (bRSV) have also been identified (e.g. A51908 and 391-2).

hRSV genomic RNA is approximately 15.2 kb in length. Transcription of the genome initiates at the 3' extracistronic region and proceeds in a sequential polar fashion to yield 10 mRNAs each encoding a major polypeptide. The hRSV genome also has a 44 nucleotide (nt) leader at the 3' end and a 155 nt noncoding trailer sequence at the 5' end (Mink, M.A., et al., (1991) *Virology* 185, 615-624). Proceeding from 3' to 5' on the genome, wild type hRSV includes the following 10 genes: NS1 and NS2 (also referred to as 1C and 1B), which encode two non-structural proteins ; N, which encodes the nucleocapsid protein; P, the phosphoprotein; M, the matrix protein; SH, a small hydrophobic protein; G, the attachment glycoprotein; F, the fusion protein; 22K, a second matrix-like protein and L, which encodes the RNA-dependent, RNA polymerase.

Complete nucleotide sequences have been determined for the nine smaller RSV genes (Collins, Peter L., (1991) *The Molecular Biology of Human Respiratory Syncytial Virus (RSV) of the Genus Pneumovirus* in The Viruses, Frankel Conrat & Kobert Wagner (ed. David Kingsbury Plenum, New York; Collins, P.L. et. al., (1991) *Proc. Natl. Acad. Sci. USA 88*:9663-9667; Sullender , W.M. et. al., (1991) *J. of Virology 65:* 5425-5434; Sullender, W.M. et. al., (1990) *Virology 178*:195-203; Collins, P.L. and G.W. Wertz, (1985) Virology 141:283-291; P.L. Collins and G.W. Wertz,(1985) *J. of Virology 54*:65-71; Collins, P.L. and G.W. Wertz (1985) *Virology 143*:442-451; Collins, P.L. et. al., (1985) *Virology 146*: 69-77; Collins, P.L. et. al., (1984) *J. of Virology 49:* 572-578; Satake, M. et. al., (1984) *Journal of Virology 52*: 991-994; Collins, P.L. and G.W. Wertz (1983) *Proc. Natl. Acad. Sci. USA 80*: 3208-3212). In addition, a functional cDNA encoding functional RNA-dependent RNA polymerase was identified as described in the Example. This novel cDNA is disclosed herein as SEQ ID NO: 1. Modifications (e.g. base substitutions) of this exact nucleotide sequence

can be performed by one of skill in the art and modified sequences can be tested for functional activity using the system for recovering replicable RS virus RNAs entirely from cDNA clones as described in Example 1.

The bRSV genome encodes 10 proteins that correspond closely in size to the hRSV proteins (Lerch, R. A., (1989) *Journal of Virology*, 63, 833-840). Complete nucleotide sequences have been determined for the N (Amann, V. L., (1992) *Journal of General Virology*, 73 999-1003); F (Lerch, R. A., et al., (1991) *Virology*, 181, 118-131) and G (Lerch, R. A. et al., (1990) *Journal of Virology*, 64, 5559-5569) proteins. cDNA clones corresponding to 9 of the 10 bRSV mRNAs (all but the L protein) have been constructed (Lerch, R. A. et al., (1989) *Journal of Virology*, 63, 833-840).

Although infectious respiratory disease caused by hRSV infection is responsible for an estimated 2.2 million human deaths annually, the majority in infancy (Pringle, C.R. (1991) *Bulletin of the World Health Organization 65*:133-137), and bRSV epidemics in cattle (particularly in winter) are of economic significance to the beef industry (Bohlender, R.E., et al., (1982) *Mod. Vet. Pract.* 63, 613-618; Stott, E.J. and G. Taylor, (1985) *Arch. Virol*, 84, 1-52; Stott, E.J., et al., (1980) *J. Hyg.* Vol. 85, 257-270), no effective vaccine against hRSV or bRSV is yet available.

This unfortunate situation is compounded by the fact that maternal antibodies do not confer solid immunity on neonates (Stott, E.J. et. al., (1987) *Journal of Virology 60*, 607-613) and natural infection affords only partial protection against frequent repeat infections, as immunity to hRSV is complex, involving both antibody and cell-mediated response (Stott, E.J and G. Taylor (1989) Immunity to Respiratory Syncytial Virus p. 85-104. In Immune Responses, Virus Infections and Disease, N.J. Dimmock, and P.D. Minor, (ed.), vol. 27. IRL Press, Oxford).

A disturbing aspect of the immune pathology of hRSV induced respiratory disease was revealed when a formalin inactivated vaccine was tested. Although the vaccine was antigenic and elicited neutralizing antibody, it failed to protect against subsequent infection, and in fact, its use resulted in enhanced frequency and severity of lower respiratory tract disease in children exposed to subsequent reinfection (Fulginiti, V.A. et. al., (1969) *American Journal of Epidemiology 89*, 435-448 and Kim, H et. al., (1969) *American Journal of Epidemiology 89*, 422-434). It is still unclear why the formalin inactivated live virus vaccine failed.

Naturally attenuated RSV vaccines have been prepared (for example by serially passaging virulent respiratory syncytial virus in human diploid lung fibroblasts see U.S. Patent Nos. 4,122,167 and 4,145,252 to Buynak and Hilleman; and/or by cold-passage or introduction of mutations which produce viruses having a temperature sensitive or cold adapted phenotype see WO 93/21320 to Murphy et. al.). However, attenuated RSV live virus vaccines have proven to be poorly infectious and overall ineffective in the prevention of respiratory syncytial virus mediated disease.

To address this major health problem, work over the past ten years has focused on the molecular biology of hRSV. cDNAs to all of the RS virus mRNAs have been characterized and used to demonstrate that the negative strand RNA genome of the RS virus possesses 10 genes encoding 10 unique polypeptides (Collins, P.L., Huang, Y.T. and G.W. Wertz (1984) *Journal of Virology 49*, 572-578). The possession of 10 genes sets RS virus apart from other paramyxoviruses, which have only six or seven genes. The RS virus genes, proceeding in order from 3' to 5' on the genome are: NS1 and NS2, which encode two non-structural proteins; N, which encodes the nucleocapsid protein; P, the phosphoprotein; M, the matrix protein; SH, a small hydrophobic protein; G, the attachment glycoprotein; F, the fusion protein; 22K, a second matrix-like protein and L, which encodes the RNA-dependent, RNA polymerase.

Based on the identification of RSV genes and encoded proteins, a variety of vaccines have been prepared. For example, U.S. Patent No. 5,149,650 by Wertz et. al., describes hRSV subunit vaccines comprising recombinant human RSV (rhRSV) structural proteins. U.S. Patent No. 5,223,254 by Paradiso et. al., describes rhRSV subunit vaccines comprising polypeptides related to a neutralizing epitope, a fusion epitope, or both, of RS virus glycoproteins, including the F and/or G protein of hRSV, as well as viral vaccines encoding the polypeptides. U.S. Patent No. 5,288,630 by Wathen et. al., describes vaccines made from DNA viruses such as vaccinia expressing an FG rhRSV chimeric protein. However, none of the currently available vaccines have proven to be both safe and effective at immunizing a subject against RSV infection.

Recombinant DNA techniques (including the use of site specific mutagenesis) offer the possibility of designing highly effective vaccines based on RSV whole or partial viral genomes. However, the RNA of negative stranded viruses is not by itself competent to initiate infection or replication (Huang, Y.T., Collins, P.L. and G.W. Wertz (1985) *Virus Research 2*, 157-173). In virions or intracellularly, RSV RNA is always found tightly encapsidated in a ribonucleoprotein core. This nucleocapsid provides the proteins necessary for transcription and replication and is the minimal unit of infectivity.

Although one group has used recombinant techniques to produce synthetic RSV particles from cDNA (Collins, P.L., et. al., (1991) *Proc. Natl. Acad. Sci. USA 88,* 9663-9667), wild type hRSV helper virus was used to provide the proteins required for transcription and replication. Contamination by the wild type helper virus, however, makes this method unsuitable for RSV vaccine preparations. In addition, this system works at low efficiency, so that a reporter gene or strong positive selection is required to detect expression from a virus containing the rescued RNA.

The inventors and co-workers have described a method for recovering an infectious 2.2kb defective interfering particle of vesicular stomatitis virus (VSV) from a cDNA clone by a method that does not require the presence of wildtype helper virus (Pattnaik, A.K. et. al., (1992) *Cell 69*:1011-1020).

A means for generating non-segmented, negative sense virus particles that are not contaminated by wild type helper virus would be useful for producing safe and effective vaccines, gene therapy vectors, and antiviral agents.

**Summary of the Invention**

In a first aspect, the invention features negative stranded, non-segmented virus particles, which can be formulated as vaccines, gene therapy vectors or anti-viral agents. At least three different categories of particles can be made, each depending on the inclusion or exclusion of viral genes required for various steps in the replication process (i.e., transcription, genome replication, encapsidation, assembly and release of infectious particles).

One type of non-segmented virus particle, a *replicating, spreading virus particle*, comprises: i) a non-segmented virus RNA dependent RNA polymerase (L); ii) a non-segmented virus phosphoprotein (P); iii) a non-segmented virus nucleocapsid (N); iv) necessary non-segmented virus structural proteins; v) a 3' non-coding RNA sequence; vi) a 3' to 5' RNA coding region, which encodes the viral proteins required to support viral particle transcription and replication in a newly infected cell and production and assembly of budded infectious particles (i.e. (i) - (iv) above) and optionally includes a heterologous gene (X); and vii) a 5' non-coding RNA sequence. Since these particles can infect cells, replicate their genome, transcribe encoded gene(s), and produce and assemble budded infectious particles, they can effect a long- lasting immunity or gene therapy in a subject.

Another non-segmented virus particle, a *replicating, non-spreading virus particle*, comprises: i) a non-segmented virus L protein; ii) a non-segmented virus P protein; iii) a non-segmented virus N protein; iv) necessary non-segmented virus structural proteins; v) a 3' non-coding RNA sequence; vi) a 3' to 5' RNA coding region, which encodes the viral proteins required to support viral particle transcription, replication and nucleocapsid assembly in a newly infected cell, but not production and assembly of budded infectious particles (i.e. (i)- (iv) above), and optionally includes a heterologous gene (X); and vi) a 5' non-coding RNA sequence. These particles can infect cells, replicate their genome and transcribe encoded gene(s), which can then be expressed in that cell. However, because they do not encode structural proteins required to produce and assemble budded infectious particles, the particles are incapable of budding off virions and spreading to other cells. These particles are particularly useful as vaccines or gene therapy vectors in applications where it is desirable to control (limit) expression of encoded genes (e.g. antigenic or therapeutic proteins or peptides) by controlling the number of cells infected.

A further non-segmented virus particle, a non-segmented virus *transcribing particle,* comprises: i) a non-segmented virus L protein; ii) a non-segmented virus P protein; iii) a non-segmented virus N protein; iv) necessary non-segmented virus structural proteins; v) a 3' non-coding RNA sequence, vi) a 3' to 5' RNA coding region which contains an appropriate transcription initiation sequence and a heterologous gene (X); and vii) a 5' non-coding RNA sequence. These transcribing particles can transcribe the heterologous gene, but can not replicate in or kill host cells. These particles can therefore be safely used as vaccines and gene therapeutics. In a preferred embodiment, the 3' noncoding sequence is the complement of the 5' non-coding sequence, so that these particles can out-compete wild type virus for proteins required for transcription and replication and therefore can be administered to a subject, for example, as an antiviral agent.

In another aspect, the invention features a novel cDNA encoding a functional respiratory syncytial virus (RSV), RNA dependent, RNA polymerase (L) protein. This cDNA has utility not only in generating recombinant RSV particles, but also in drug screening assays to identify drugs that specifically inhibit or interfere with RSV L protein function and that therefore would function as highly effective antiviral therapeutics for treating respiratory syncytial virus infection.

Recombinant, non-segmented negative stranded virus particles made as described herein are "pure" (i.e., not contaminated by negative strand helper virus). In addition, various types of particles can be formulated in accordance with the intended use. For example, replicating, spreading particles can be formulated and used as vaccines or gene

therapy vectors, where widespread and sustained expression of antigenic or therapeutic proteins is desired. Alternatively, replicating, non-spreading particles can be used as vaccines or gene therapy vectors, where limited or controlled expression of antigenic or therapeutic proteins is desired. Transcribing virus particles, on the other hand, can be administered as transient vaccine or gene therapy vectors or as anti-viral agents to interfere and prevent replication of wild-type virus.

Further, particles can be formulated to comprise (and encode) particular non-segmented, negative stranded virus proteins, for example, to optimize target cell specificity or to better accomodate particular heterologous genes. For example, particles comprised of the vesicular stomatitis virus (VSV) glycoprotein (G) proteins can infect an extremely broad range of animal cells, while particles comprised of Respiratory Syncytial Virus (RSV) G proteins specifically infect lung epithelia. Other features and advantages will be readily apparent from the following detailed description and claims.

**Brief Description of the Drawings**

Figure 1 is a diagrammatic representation of a process for generating replicating, non-spreading Respiratory Syncytial virus (RSV) particles.

Figure 2 is a diagrammatic representation of a process for generating replicating and spreading RSV particles.

Figure 3 is a diagrammatic representation of an RSV cDNA wildtype replicon.

Figure 4 is a diagrammatic representation of an RSV cDNA panhandle replicon, which can be used in making transcribing particles.

Figure 5 is a diagrammatic representation of a process for generating recombinant Vesicular Stomatitis Virus (VSV) particles by transfecting the genome into cells expressing only the three genes, N, P and L. The other genes are encoded in the replicon (pVSV).

Figure 6 is a schematic representation of the pVSV1(+) replicon and its T7 transcript.

Figure 7 is a diagrammatic representation of the genome of various VSV particles.

## Detailed Description of the Invention

### Definitions

As used herein, the following terms and phrases shall have the meanings set forth below:

A "heterologous gene (X)" refers to a nucleic acid molecule that is desired to be transcribed or expressed (i.e. transcribed and translated) from a non-segmented, negative stranded RNA virus particle. As described further below, for vaccine formulations, the heterologous gene preferably encodes a protective epitope of a pathogenic organism. For gene therapy formulations, the heterologous gene preferably encodes a protein that supplements a defective (e.g. mutant) or inappropriately expressed protein in a patient or is an antisense or other biologically active nucleic acid molecule.

A "non-segmented, negative stranded RNA virus" or "non-segmented virus" shall refer to a virus, which contains a negative sense (3'-5') non-segmented RNA genome. Non-segmented viruses are typically classified in the order Mononegavirales, which includes the Paramyxoviridae, Rhabdoviridae and Filoviridae (See Background of the Invention).

"pure" shall mean not contaminated by wild-type virus.

"recombinant" refers to generation by recombinant DNA technology.

A "replicating spreading particle" shall refer to a particle comprised of a non-segmented negative stranded RNA virus genome surrounded by non-segmented negative stranded virus proteins. The particle can enter a cell, transcribe encoded genes to yield messenger RNA (mRNA) to generate proteins, replicate the genomic RNA to produce more genomes and from them to produce more mRNA transcripts and assemble the genomes with proteins to produce viral particles which can then spread to other cells for expanded delivery.

A "replicating non-spreading particle" shall refer to a particle comprised of a non-segmented negative stranded RNA virus genome (which is incomplete) surrounded by non-segmented negative stranded virus proteins. The particle can enter a cell, transcribe encoded genes to yield messenger RNA (mRNA) to generate proteins, replicate the genomic RNA to produce more genomes and from them to produce more mRNA transcripts and

assemble the genomes with the proteins to produce viral particles which can not spread to other cells because essential genes for assembly have been omitted from that genome.

A "transcribing particle" shall refer to a particle comprised of cDNA, which includes a heterologous gene and an appropriate transcription initiation sequence and is surrounded by non-segmented negative stranded virus proteins. The particle can infect cells and transcribe an encoded heterologous gene to produce messenger RNAs for expression in that cell, but which cannot replicate to produce more genomes and can not assemble and spread to other cells, because genes for replication and assembly are not included in the cDNA.

In general, replicating and transcribing non-segmented negative strand RNA virus particles can be generated by introducing into a host cell cDNAs which minimally express the following proteins: i) a non-segmented virus RNA dependent RNA polymerase (L) protein, ii) a non-segmented virus nucleocapsid (N) protein; and iii) a non-segmented virus phosphoprotein (P). Preferably, genes encoding the L, N, and P proteins have been introduced into host cells as plasmids under the control of a promoter region that is recognized by a DNA dependent RNA polymerase, which is native to or has been engineered into the host cell. Into the same host cell is introduced a cDNA plasmid which expresses a non-segmented negative strand genome minimally containing the cis acting signals for RNA replication and transcription (a replicon).

Figure 1 is a diagrammatic representation of a process for generating RSV ribonucleoprotein (RNP) particles, which are capable of replication, but not of budding virions. Additional non-segmented virus structural proteins can be expressed in host cells in the same manner that non-segmented virus N, P, and L proteins are supplied. Alternatively, additional non-segmented virus structural proteins can be encoded in the cDNA encoding the replicon. A preferred method for making these particles is described in detail in the following Example 1.

In order to make particles that are capable of budding and forming infectious progeny virions, necessary non-segmented virus proteins must also be encoded in the replicon and expressed in a host cell. Preferred RSV structural proteins are selected from the group consisting of RSV N, P, M, M2, SH, G or F proteins. Preferred rhabdovirus (e.g., VSV or rabies virus (RV)) structural proteins include M or G proteins. Figure 2 is a diagrammatic representation of a process for generating pure, infectious and budding non-segmented viral particles.

As shown in Figures 1 and 2, in a preferred method, cDNA encoding the T7 RNA polymerase is introduced into a host cell using the vaccinia virus-T7 RNA polymerase recombinant (Fuerst, T.R. et al., (1986) *Proceedings of the National Academy of Sciences USA* 83, 8122-8126). Plasmids encoding functional proteins (N, P and L) alone (Figure 1) or in conjunction with structural proteins (Figure 2) under the control of the T7 promoters are then transfected into the host. Replicons under the control of similar T7 promoters are also transfected into the host cell.

The T7 RNA polymerase transcribes from transfected plasmids the mRNAs to be translated to yield the functional proteins and structural proteins from the replicon cDNA, genomic RNAs with precise termini, which are competent to be replicated and encapsidated to form nucleocapsids. Preferably antigenomic RNAs are expressed in the presence of a pool of nucleocapsid protein and phosphoprotein (i.e. N and P in approximately a **2:1** molar ratio) such that encapsidation of the nascent RNA can begin immediately, thereby enhancing the formation of functional ribonucleoproteins consisting of the RNA and N, P and L proteins.

In a preferred embodiment, the T7 RNA polymerase is expressed from the vaccinia virus strain MVA/T7 Pol recombinant, a highly attenuated and avian host-restricted vaccinia virus recombinant that encodes the T7 polymerase gene (Wyatt, Moss and Rosenblatt, 1995, Virology 210:202-205). Such a vaccinia recombinant is unable to replicate in mammalian cells and hence recovery of viruses from cDNA clones is free not only of helper virus, but also of the recombinant T7 expressing vector.

Figures 3 and 4 provide diagrammatic representations of RSV replicons; wildtype (Fig. 3) and panhandle (Fig. 4). For use in the invention, a replicon must include: i) a 3' non-coding RNA sequence, ii) a 3' to 5' RNA coding sequence, and iii) a 5' non-coding sequence. The 3' and 5' non-coding RNA sequences are essential to replication by a non-segmented viral polymerase. As shown in Figures 3 and 4, the 5' non-coding sequence can be a trailer sequence (e.g. the RSV 155 nucleotide trailer sequence) and the 3' non-coding sequence can be a leader sequence (e.g. the RSV 44 nucleotide trailer sequence). In general, polymerases of non-segmented viruses are specific to their own leader and trailer sequence.

The replicons shown in Figures 3 and 4 employ three basic elements for ensuring intracellular transcription of RNAs with precise termini. A truncated form of the bacteriophage T7 promoter ($\phi$10) immediately followed by a blunt end cloning site with two blunt-ended restriction sites. The promoter distal site is immediately followed by a cDNA copy of the autolytic ribozyme from the antigenomic strand of hepatitis delta virus (HDV) which, in turn, is immediately followed by a T7 terminator element (T$\phi$). Transcription by

the T7 polymerase yields an RNA with two additional nucleotides at the 5' end of the transcript, continuing through the ribozyme and terminating in the Tϕ terminator sequence. A precise 3' terminus is generated by the autolytic cleavage of the primary transcript by the HDV genomic RNA at the exact terminus of the RS virus genomic insert.

In between the 3' and 5' non-coding RNA sequences, a replicon contains a 3' to 5' RNA coding region, which includes the viral genes required to support the viral particle transcription, replication and assembly in a newly infected cell plus any heterologous gene (X) desired to be expressed. Each gene encoded in a replicon must have appropriate transcription start and stop signals and intercistronic junctions to signal transcription by the polymerase and subsequent translation to yield protein. Theoretically, there is no limit in the amount of RNA that can be included in the 3' to 5' coding region. In practice, the size of the coding cDNA will be limited by the amount that can be replicated.

An important and essential technical feature for recovering replicating spreading or replicating non-spreading virus particles, in which the 3'-5' coding region comprises a substantial portion of a non-segmented negative stranded RNA virus is the finding that recovery of a complete virus genome into virus particles could only be achieved by expressing a positive (antigenomic) sense copy of the viral RNA, rather than a negative genomic sense RNA as would be expected for a negative sense virus. The requirement for an antigenomic copy may be due to the fact that the RNA polymerase that synthesizes the initial viral RNA in the cell, terminates at each intergenic junction when transcribing a negative sense RNA. However, the polymerase does not terminate at these junctions when transcribing a positive sense RNA. Whether a particular 3'-5' coding region comprising a replicating spreading or replicating non-spreading virus particle must be antigenomic can be determined empirically as described in the following Example 2.

Another technical feature involves the ability to regulate levels of expression of foreign genes inserted into negative strand virus-based vectors by virtue of the location of the genes in the replicon. Control of gene expression in negative strand RNA viruses is a result of a single polymerase entry site at the 3' end of the genome and polymerase dissociation at each intergenic junction. Hence, genes located closest to the 3' end of the genome are transcribed in the greatest amounts and there are decreasing levels of gene expression with increasing distance of a particular gene from the 3' end of the genome. Therefore levels of expression can be increased or decreased by altering the location of the foreign gene insertion relative to the genomic 3' end. Preferred sites of insertion in a VSV genome are shown in Figure 7.

Eukaryotic cells are preferable "host cells" for producing non-segmented viral particles in vitro. Preferred host cells are mammalian cell lines which are capable of being infected by a non-segmented virus (e.g. HEp-2, HeLa, thymidine kinase deficient (tk-) cells, human embryonic diploid fibroblasts, primary monkey or calf kidney cells, human embryonic kidney, COS, C127, baby hamster kidney (BHK), Vero, LLCMK-2, BSC-1, CV-1, 293 and CHO cells. Non-segmented virus particles comprised of VSV proteins grow to high titers in most animal cells and therefore can be readily prepared in large quantities.

Introduction of replicons into a host cell can be accomplished using standard techniques (e.g. via viral infection, calcium phosphate or calcium chloride co-precipitation, DEAE dextran mediated transfection, lipofection or electroporation). A preferred method of introduction is described in Fuerst, T.R. et. al., (1987) Use of a Vaccinia Virus T7 RNA Polymerase System for Expression of Target Genes. *Mol. Cell. Biol.* 7:2538-44. Cells expressing the infectious viral particles can be cultured in vitro and the particles can be purified using well-known techniques.

As an alternative to production of non-segmented viral particles by in vitro culture, the particles can also be produced in vivo, for example by introducing appropriate expression systems into an animal host having cells that are capable of being infected by the virus and contain (or have been engineered to contain) functional, non-segmented virus L, N and P proteins.

*Vaccines*

Using the above-described system for replicating pure populations of infectious non-segmented virus particles, a variety of vaccines can be formulated and administered to a subject to induce an immune response against any of a number of pathogenic infections. Preferred vaccine particles include structural proteins obtained from a non-segmented negative stranded virus that is a non-human pathogen and non-oncogenic (e.g. VSV).

The 3' to 5' coding region of appropriate vaccine candidates will include at least one heterologous gene (X) encoding a protective epitope (i.e. an epitope that elicits an immune response in a subject) of a pathogenic bacterium, virus (e.g. HIV, herpes, hepatitis, RSV, parainfluenza virus 3, measles, mumps, rabies, Ebola, Hanta) fungi (e.g. *Candida sp.* ) or protozoan (e.g. *Toxoplasma gondii*, ). Table 1 sets forth a representative list of pathogens against which non-segmented viral particle vaccines can be prepared.

Table 1
Candidate Pathogens for Vaccine Development

| PATHOGEN | POTENTIAL EFFECTS | CASES PER YEAR (AND DEATHS) | INDUSTRIAL DEMAND |
|---|---|---|---|
| Dengue virus | Fever, shock, internal bleeding | 35,000,000 (15,000+) | Small, (travelers to endemic areas) |
| Intestinal-toxin-producing *Escherichia coli* bacteria | Watery diarrhea, dehydration | 630,000,000 (775,000+) | Small |
| *Hemophilus influenzae* type b bacterium | Meningitis, epiglottal swelling, pneumonia | 800,000 (145,000+) | Great |
| Hepatitis A virus | Malaise, anorexia, vomiting, jaundice | 5,000,000 (14,000) | Small |
| Hepatitis B virus | Same as hepatitis A; Chronic cirrhosis or cancer of liver | 5,000,000 (822,000) | Moderate |
| Japanese encephalitis virus | Encephalitis, meningitis | 42,000 (7,000+) | Small (Travelers) |
| *Mycobacterium leprae* | Leprosy | 1,000,000 (1,000) | None |
| *Neisseria meningitidis* bacterium | Meningitis | 310,000 (35,000+) | Some (during epidemics) |
| Parainfluenza viruses | Bronchitis, pneumonia | 75,000,000 (125,000+) | Great |
| *Plasmodium* protozoa | Malaria (with anemia, systemic inflammation) | 150,000,000 (1,500,000+) | Moderate (travelers) |
| Rabies virus | Always-fatal meningitis and encephalitis | 35,000 (35,000+) | Small |
| Respiratory syncytial virus | Repeated respiratory infections, pneumonia | 65,000,000 (160,000+) | Great |
| Rotavirus | Diarrhea, dehydration | 140,000,000 (873,000+) | Great |
| *Salmonella typhi* bacterium | Typhoid fever (with platelet and intestinal damage possible) | 30,000,000 (581,000+) | Small (travelers) |
| *Shigella* bacteria | Diarrhea, dysentery, chronic infections | 250,000,000 (654,000+) | None |
| *Streptococcus* Group A bacterium | Throat infection, then rheumatic fever, kidney disease | 3,000,000 (52,000+) | Small |
| *Streptococcus pneumoniae* bacterium | Pneumonia, meningitis, serious inflammation of middle ear | 100,000,000 (10,000,000+) | Small to moderate |
| *Vibrio cholerae* bacterium | Cholera (with diarrhea, dehydration) | 7,000,000 (122,000+) | Small (travelers) |
| Yellow fever | Fever, jaundice, kidney damage, bleeding | 85,000 (9,000+) | Small (travelers) |

Alternatively, non-segmented virus particles can be used to infect an appropriate host cell (in vitro or in vivo) for production of recombinant pathogen protective epitopes, which can then be formulated into a "subunit vaccine".

An "effective amount" of live-virus or subunit vaccine prepared as disclosed herein can be administered to a subject (human or animal) alone or in conjunction with an adjuvant (e.g. as described in U.S. Patent 5,223,254 or Stott et al., (1984) *J. Hyg. Camb.* 251-261) to induce an active immunization against a pathogenic infection. An effective amount is an amount sufficient to confer immunity against the pathogen and can be determined by one of skill in the art using no more than routine experimentation. Determination of an effective amount may take into account such factors as the weight and/or age of the subject and the selected route for administration.

A cocktail of infectious virus particles expressing various pathogen protective epitopes can also be prepared as a vaccine composition. Vaccines can be administered by a variety of methods known in the art. Exemplary modes include oral (e.g. via aerosol), intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, parental, transdermal and intranasal routes. If necessitated by a particular mode, the vaccine may be encapsulated.

*Gene Therapy Vectors*

An important application of this technology is the use of non-segmented virus particles for the transcription or expression of heterologous genes (X) from host (e.g., human or animal) cells. Based on the work described in the following examples, it would appear that even very large genes can be accommodated in non-segmented virus particles. VSV based particles are particularly suitable for accomodating large inserts, since VSV has a helical ribonucleocapsid in which there is a linear relationship between genome length and particle size, suggesting that packaging constraints on the amount of additional nucleic acid are minimal.

Furthermore, the levels of expression of foreign genes in non-segmented virus particles can be regulated both by their location within the genome, as explained above, and by altering the adjacent cis-acting sequences that function as promoters. The following Table 2 is a representative list of genes that can be administered to a subject via non-segmented virus particles to treat a disease.

Table 2
Gene Therapy

| Disease | Therapeutic Gene/Protein |
|---|---|
| **IMMUNE DEFICIENCIES** | |
| adenosine deaminase deficiency | adenosine deaminase |
| purine nucleoside phosphorylase deficiencies | purine nucleoside phosphorylase |
| osteoporosis | carbonic anhydrase II |
| **HEMATOPOIETIC DISORDERS** | |
| anemia | erythropoietin |
| thalassemia | α,β thalassemia |
| thrombopenia | thrombopoietin |
| sickle cell disease | anti-sickling globin |
| **SERUM PROTEIN DEFICIENCIES** | |
| hemophilia (A & B) | factor VIII and<br>factor IX |
| α-1-antitrypsin deficiency | α-1-antitrypsin |
| hereditary angioneurotic edema | C1 esterase inhibitor |
| | |
| **INBORN METABOLISM ERRORS** | |
| urea cycle metabolism | carbamyl phosphate synthetase, ornithine transcarbamylase, argininosuccinate lyase, arginase |
| organic disorders | propionyl CoA carboxylase,<br>methylmalonyl CoA mutase |
| phenylketonuria | phenylalanine hydroxylase |
| galactosemia | galactose-1-phosphate uridyl transferase |
| homocystinuria | cystathionine β-synthase |
| maple syrup urine disease | branched chain 2-keto acid decarboxylase |

Table 2 ( continued)

| Disease | Gene |
|---|---|
| **STORAGE DISEASES** | |
| Fabry's disease | galactosidase |
| Gaucher's disease | glucocerebrosidase |
| **CNS DISORDERS** | |
| Lesch-Nyhan syndrome | hypoxantihine phosphoribosyl transferase |
| Tay-Sachs disease | hexosaminidase |
| **FAMILIAL HYPERCHOLESTEROLEMIA** | |
| familial hypercholesterolemia | low-density lipoprotein receptor |
| **ENDOCRINE DISORDERS** | |
| diabetes mellitus | insulin |
| hypopituitarism | growth hormones; growth factors |
| **IMMUNOLOGIC DISORDERS** | |
| lymphokine deficiencies | interleukins; interferons; cytokines; colony stimulating factors |
| **OTHER** | |
| Cystic Fibrosis | cystic fibrosis transmembrane conductance regulator protein |
| Duchenne muscular dystrophy | dystrophin |
| cancer, tumors, pathogenic infections | antibodies; antibacterial, antiviral, anti-fungal and antiprotozoal agents; multidrug resistance and superoxide dismutase |
| wound healing | transforming growth factors |

Alternative to encoding proteins or peptides, non-segmented virus gene therapy vectors can contain antisense oligonucleotides or other nucleic acid biological response modifiers.

A particular non-segmented virus particle can be selected for a particular gene therapy based on the tropism of the natural, wildtype virus. For example, with VSV, target cell specificity is mediated by the attachment of glycoprotein G, which permits the infection of virtually all animal cells that have been studied.

Natural respiratory syncytial virus specifically, on the other hand, only infects respiratory tract tissue (e.g. lung epithelia). Based on this natural affinity, RSV particles can

be used as gene therapy vectors for delivery to a subject's respiratory tract. In a preferred embodiment, the protein expressed from an RSV based particle has bioactivity in a subject's lung. In a particularly preferred embodiment, the protein is selected from the group consisting of: the cystic fibrosis transmembrane conductance regulator (CFTR) protein or a functional fragment thereof, an anti protease (e.g. alpha-1-antitrypsin), a tissue inhibitor of metaloproteinase, an antioxidant (e.g., superoxide dismutase), a cytokine (e.g., an interferon), a mucolytic (e.g., DNAse); or a protein which blocks the action of an inflammatory cytokine.

An "effective amount" of a gene therapy vector prepared from a non-segmented viral particle can be administered to a subject (human or animal). An effective amount is an amount sufficient to accomplish the desired therapeutic effect and can be determined by one of skill in the art using no more than routine experimentation. Determination of an effective amount may take into account such factors as the weight and/or age of the subject and the selected route for administration.

Gene therapy vectors can be administered by a variety of methods known in the art. Exemplary modes include oral (e.g. via aerosol), intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, parenteral, transdermal and intranasal routes. If necessitated by a particular mode, the gene therapy vector may be encapsulated.

In addition to being prepared as a gene therapy pharmaceutical, infectious non-segmented virus particles can be used to infect an appropriate host cell to produce the recombinant protein in vitro (e.g. in a cell culture) or in vivo (e.g. in a transgenic animal).

*Anti-viral Agents*

**transcribing particles**

Defective interfering particles are subgenomic virus particles (lacking greater or lesser percentages of the virus genome). They contain virus structural proteins and antigens. DI particles require homologous parental (wildtype) virus for replication and replicate preferentially at the expense of helper virus, thereby causing interference. Defective interfering particles can also enhance interferon production, modulate surface expression of viral proteins, affect their transport, processing and turnover, and alter the timing and basic pathology of a virus infection *in vivo* (Holland, John J., *Defective Interfering Rhabdoviruses*. Dept. of Biology, University of California at San Diego, La Jolla, California 92093. Chapter 8, pp. 297-360).

As described in detail in the following Example, defective interfering-like particles, replicating particles have been made using the panhandle RSV replicon shown in Figure 4. The panhandle construct contains an authentic 5' terminus and its complement at the 3' terminus as found in copy-back DI RNAs of other negative strand viruses. These replicating particles can out-compete wild type virus for proteins required for transcription and replication and therefore can be administered to a subject as an antiviral agent.

Other replicating and transcribing particles can comprise: i) a non-segmented virus L protein; ii) a non-segmented virus P protein, iii) a non-segmented virus N protein, iv) a 3' non-coding RNA sequence, v) a 3' to 5' RNA coding region, which contains an appropriate transcription initiator and encodes a heterologous gene, and vi) a 5' non-coding RNA sequence can be designed. Preferable replicating and transcribing particles, (i.e. transcribing particles with the greatest replicative advantage) maximize the extent of terminal complementarity between the 3' and 5' non-coding sequences and still maintain a transcription start site. Work with copy-back like VSV particles, has shown that the extent of complementarity, rather than their exact sequence, is a major determinant of whether a template predominantly directs transcription or replication (Wertz, G. et al., (1994) *Proc. Natl. Acad. Sci. USA*,, 91, 8587-8591).

drug screening

Effective antiviral drugs specifically prevent or neutralize viral infectivity without affecting host cells. Because the RNA dependent RNA polymerase performs a function unique to negative stranded RNA viruses, a drug that could interfere with the function would be a useful therapeutic against RSV mediated disease. Host cells expressing RSV RNA dependent RNA polymerase as described herein can be used as screens to test various drug candidates for anti- respiratory syncytial virus activity. For example, one can infect cells with VVTF7-3, transfect in the plasmids for N,P,L and suitable RSV mini genomes and measure the effect of drugs on RSV specific RNA replication and transcription, for example, using suitable radiolabelling techniques. This could be accomplished as a screen in cells in culture.

An "effective amount" of an antiviral compound, such as a defective interfering particle or drugs specifically interfering with the replication or transcription of a non-segmented virus, can be administered to a subject (human or animal). An effective amount is an amount sufficient to alleviate or eliminate the symptoms associated with viral infection. The effective amount for a particular antiviral agent can be determined by one of skill in the art using no more than routine experimentation. Determination of an effective

amount may take into account such factors as the weight and/or age of the subject and the selected route for administration.

Antiviral agents can be administered by a variety of methods known in the art. Exemplary modes include oral (e.g. via aerosol), intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, parental, transdermal and intranasal routes. If necessitated by a particular mode, the gene therapy vector may be encapsulated.

The present invention is further illustrated by the following examples, which should not be construed as limiting in any way. The contents of all cited references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference.

## Example 1 Functional cDNA Clones of RSV N, P and L Proteins Support Replication of RSV Genomic RNA Analogs and Define Minimal *Trans*-acting Requirements for Replicating

**Materials and Methods**

*Construction of full length cDNAs encoding the RS virus N, P and L proteins*

All procedures and reaction conditions for plasmid constructions were carried out according to standard methods (Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). The plasmid constructs were verified by DNA sequence determination of the relevant regions by the dideoxy chain termination method using denatured plasmid DNA as templates (Haltiner, M. et al., (1985) *Nucleic Acids Research* 1015-1028).

In order to express RS virus proteins in the VVT7 based reverse genetic analysis system, cDNA clones of the RS virus N, P and L genes were cloned into pGEM3 vectors downstream of the T7 RNA polymerase promoter, the clones were designated pRSV-N, pRSV-P and pRSV-L, respectively. Briefly, pRSV-N was prepared by transferring a BamHI-PstI fragment containing the entire N gene from pAQ330 (King et al., (1987) *Journal of Virology* 61, 2885-2890) into a pGEM3 vector. cDNA encoding the P protein was generated by reverse transcription of RS virus genomic RNA, followed by PCR amplification with a pair of oligonucleotide primers corresponding to nt positions 2328-2349 and 3459-3443 of the genome (Galinski 1991), respectively. The cDNA was then cloned into the KpnI-BamHI

site of pGEM3. Because of the size of the L gene, (6,578 nt, Stec et al., (1991) *Virology* 183, 273-287), the full-length L gene clones were constructed through several steps of subcloning and finally by assembling four exchangeable segments. Segment 1 (KpnI-MspI fragment, positions 1-1906 in the L gene), segment 2 (MspI-PflMI fragment, positions 1907-3795) and segment 4 (MunI-PstI fragment, positions 5547-6732) were prepared by reverse transcription and PCR amplification, using three pairs of oligonucleotide primers corresponding to nt positions 1-17 and 1923-1903, 1881-1902 and 3802-3788, and 5420-5441 and 6732-6700 of the L gene, respectively. Segment 3 (PflMI-MunI fragment) came from an existing clone pRSVL-35 which was prepared by oligo-dT primed cDNA synthesis (Collins and Wertz (1983) *Proceedings of the National Academy of Sciences*, USA 80, 3208-3212). The originally assembled clone yielded a 170 KDa polypeptide on translation. Sequencing analysis revealed that an adenosine residue at nt position 4762 of the L gene had been deleted, resulting in a frame-shift generating a premature stop codon 48 nt downstream of the deletion. This sequence error was repaired by site directed mutagenesis.

*Generation of cDNA clones encoding RS virus genomic analogs*

cDNA clones that transcribe two types of RS virus genomic analogs were constructed. The first type (wild-type) contained the authentic 3' and 5' termini of the genome, but deleted the majority of the internal genes, and the second type (Panhandle-type) contained complementary termini, derived from the 5' terminus of the genome, surrounding a partial L gene. Diagrams of these two analogs are shown in Figs. 1 and 2.

The wild -type analog plasmid (pWT) was prepared as follows: cDNA containing the 3' leader, 1C, 1B, N and part of P genes was synthesized by reverse transcription and followed by PCR amplification with a pair of oligonucleotide primers corresponding to nt positions 1-29 and 2378-2360 of the genome. A 2.3 kb PCR product was cloned into the KpnI-SalI site of pGEM3, and the resulting plasmid was digested with SacI and MunI to release a 0.4kb fragment containing the 3' 44-nt leader and nucleotides 1-375 of the 1C gene. Consequently, this SacI-MunI fragment was fused with a MunI-PstI fragment containing nucleotides 5547-6578 (1031-nt) of the L gene and 155-nt trailer, which was derived from pRSV-L, and cloned into pGEM3. The resulting clone was digested with BsiWI and the termini of the released 1.6kb BsiWI fragment repaired by partial filling with dGTP, dTTP, Klenow (BRL), followed by mung bean nuclease digestion. This generated a blunt-ended DNA fragment whose terminal sequences precisely matched the authentic termini of the RS virus genome. This fragment was then inserted into the SmaI site of a transcription plasmid between the T7 promoter and the antigenomic-strand of hepatitis delta virus (HDV) autolytic ribozyme followed by T7 terminator sequences (Ball, L.A. et al.,

(1992) *Journal of Virology* 66, 2335-2345.). In this sequence context, the wild-type RNA analog synthesized by the T7 RNA polymerase was predicted to contain two non RS virus GTP residues at the 5' end and, after autolytic cleavage, an exact terminus corresponding to the authentic genome 3' end.

The panhandle-type analog (pPH) was derived from the trailer region and the L gene end sequences. A 1.2kb MunI-PstI fragment containing the L gene 5' end and the trailer region was isolated from pRSV-LS4 and fused with an ApoI-PstI fragment comprising 75 nucleotides of the extreme 5' end of the trailer, and subsequently cloned into the PstI site of pGEM3. The resulting clone was treated the same as described in the wild-type analog construction and finally transferred into the SmaI site of the intracellular transcription plasmid. Therefore, the T7 transcripts from the panhandle type analog plasmid were predicted to contain, after autolytic cleavage, complementary 75-nt termini derived from the trailer, surrounding the 1031-nt L gene end.

*Virus infections and DNA transfections*

293 cells were grown in Dulbecco's Modified Eagle Medium (D-MEM, GIBCO Laboratories) containing 10% heat-inactivated fetal bovine serum (FBS) in 60 mm plates. A subconfluent cell monolayer (about $3X10^6$ cells per plate) was infected with recombinant vaccinia virus vT7-3 (moi of 10 pfu per cell) that expresses T7 RNA polymerase. After 45 minutes virus absorption, the cells were washed once with D-MEM (without FBS) and then transfected with appropriate plasmid DNAs using lipofectin according to manufacturer's (Bethesda Research Laboratories) instructions. For protein expression, the cells were transfected with 5 ug of pRSV-N, pRSV-P or pRSV-L individually or simultaneously, whereas for RNA replication assay, the cells were transfected with 5 ug of pPH3 or pWT1, and combinations of 5 ug of pRSV-N, 2 ug of pRSV-P and 0.25-2.0 ug of pRSV-L. The transfected cells were then incubated in D-MEM (without FBS) 37° C for 12-16 hours before labeling with radioisotopes.

*Immunoprecipitation and electrophoretic analysis of proteins*

For radiolabeling of expressed proteins, at 12 hours posttransfection the cells were incubated in methionine-free medium (GIBCO Laboratories) for 45 minutes and then exposed to [$^{35}$S]methionine (20 uCi/ml, Du Pont/NEN) for 3 hours. Cytoplasmic extracts of cells were prepared and viral specific proteins were immunoprecipitated as described previously (Pattnaik, A. K. and G.W. Wertz. (1990) *Journal of Virology* 64, 2948-2957) by

using goat polyclonal antiserum raised against RS virus (Chemicon International). For detecting expression of the L protein, rabbit antisera raised against the L protein specific peptides (three peptides corresponding to amino acid positions 1696-1713, 1721-1733 and 2094-2110, respectively, Stec et al., (1991) *Virology* 183, 273-287) were synthesized by the UAB Protein Synthesis Core Facility, conjugated to KLH and antiserum raised in rabbit by Lampire Biological Laboratories, Inc. A combination of the anti-L-peptide sera was used for immunoprecipitation of the L protein. Immunoprecipitated proteins were analyzed by electrophoresis on 10% polyacrylamide gels and detected by fluorography as described previously (Pattnaik, A.K. and G.W. Wertz, (1990) *Journal of Virology* 64, 2948-2957).

*Analysis of RNA replication*

To analyze RNA replication, cells were exposed to [$^3$H]uridine (25 uCi/ml, Du Pont/NEN) between 16-24 hours posttransfection, in the presence, where indicated, of actinomycin D (10 ug/ml, water-soluble mannitol complex; Sigma Chemical Co.) after 30 minutes actinomycin D pretreatment. Cells were harvested and cytoplasmic extracts prepared as described previously. Either total RNA or N-protein encapsidated RNA selected by immunoprecipitation with the goat antiserum, was extracted and analyzed by electrophoresis on 1.75% agarose-urea gels and detected by fluorography (Wertz, G.W. and N. Davis (1981) *Nucleic Acids Research* 9, 6487-6503).

*RNA protection assay of replication products*

An RNA protection assay (RPA) was used to detect strand-specific RNA synthesis using an RPA II kit according to manufacturer's instructions (Ambion). Briefly, N-protein encapsided RNAs from one 60 mm plate of cells transfected with pPH3 and the N, P or N, P and L protein plasmids as described above (without radiolabeling and actinomycin D treatment) were selectively enriched by immunoprecipitation and were used in RPA for each reaction. A strand-specific RNA probe was generated by T7 RNA polymerase *in vitro* transcription of a pGEM3 plasmid with incorporation of [$^{35}$S] UTP (Du Pont/NEN) according to the manufacturer's instructions (New England Biolabs). The pGEM3 plasmid containing a Bc1I-Bg1II fragment of the L gene end (positions 5655-6514) was linearized by digestion with SspI, the cleavage site for which is present in the Bc1I-Bg1II fragment (position 6158), so that run off T7 polymerase transcription produced a 391-nt RNA probe. The RNA probe was purified by polyacrylamide gel electrophoresis. The specific activity of the purified probe was determined and $6x10^3$ cpm of probe was used in each reaction of the assay. The protected RNA was analyzed by electrophoresis on 4.5% sequencing gels and detected by fluorography.

**Results**

*Expression of RS virus Proteins*

In order to establish a reverse genetic approach for analysis of RS virus, it was necessary to prepare cDNA clones capable of expressing the RS virus proteins involved in RNA replication. By analogy with other negative-stranded RNA viruses, these would most likely be the N, P and L proteins, although at the outset it was unknown whether the nonstructural proteins 1C and 1B might also be required. Full-length cDNA clones of the N, P and L genes were prepared as described and subcloned into the expression vector pGEM3. To detect whether these cDNA clones expressed N, P and L proteins, the recombinant vaccinia virus-T7 RNA polymerase expression system was used. (Fuerst, T.R. et al., (1986) *Proceedings of the National Academy of Sciences USA* 83, 8122-8126). 293 cells were infected with vTF7-3 and transfected with plasmids pRSV-N, pRSV-P, or pRSV-L. At 12 hours posttransfection, the cells were labeled with [$^{35}$S]methionine for 3 hours. Cytoplasmic extracts were prepared, and proteins were immunoprecipitated with anti-RS virus antibody in the case of the N and P proteins, or anti-L-peptide antisera in the case of the L protein, and analyzed by electrophoresis.

vTF7-3 infected cells transfected with pRSV-N expressed a protein which comigrated with the authentic N protein synthesized in RS virus infected cells. Similarly, vTF7-3 infected cells transfected with pRSV-P also expressed a protein which comigrated with the authentic P protein. Neither untransfected nor uninfected cells produced these proteins, suggesting that pRSV-N and pRSV-P expressed the appropriate viral proteins.

A cDNA clone containing the L gene constructed as described above was transfected into vTF7-3 infected cells. The total expressed products were analyzed by SDS-PAGE and a polypeptide with a molecular weight of 170 kDa was observed, but not the expected 250 kDa polypeptide. Sequencing analysis revealed that an adenosine residue at nt position 4762 of the L gene had been deleted, resulting in a frame-shift which generated a premature stop codon 48 nt downstream of the deletion. The sequence error was repaired by restoring the A residue by site-directed mutagenesis. A corrected L gene cDNA clone was constructed and expressed in the same system. In order to detect the L protein, rabbit anti-L-peptide sera were prepared and used to immunoprecipitate the products of expression. The results showed that a polypeptide of 250 Kd expressed from the repaired L gene clone was identified by the anti-L-peptide sera, which comigrated with the authentic L protein. A few faint bands migrating faster than the L protein were also observed, which might be the

products derived from late initiations of translation, or degradation of the L protein. This work demonstrated that the corrected full-length L gene clone was capable of directing synthesis of authentic size RS virus L protein. Consequently, this cDNA clone was used in RNA replication experiments to test whether the expressed L protein was a functional polymerase.

*Expression of genomic RNA analogs*

To establish the reverse genetic analysis system, cDNA clones that transcribed two types of RS virus genomic RNA analogs were constructed. As shown in Figure 1, the wild-type cDNA clone, pWT1, encoded an analog of RS virus genomic RNA in which the majority of the internal genes were deleted. Transcription of pWT1 by T7 RNA polymerase would yield a 1605-nt long, negative-sense RNA with the authentic 3' terminus of the RS virus genome, created by the autolytic cleavage of the ribozyme, and the following structural features (listed in 3' to 5' order): (i) the 44-nt leader region; (ii) nt 1-375 of the 1C gene; (iii) nt 5547-6578 (1031-nt) of the L gene; (iv) the 155-nt trailer region and (v) two non RS virus GTP residues encoded by the vector. Similar to pWT1, the panhandle-type cDNA clone, pPH3, encoded an RS virus genomic analog in which most of the internal genes had been deleted.

However, in contrast to pWT1, pPH3 contained DI-like termini, i.e., complimentary termini surrounding a partial L gene (Figure 2). As with pWT1, the panhandle-type genomic analog sequences were also placed in the transcription plasmid under T7 promoter control and followed by the HDV ribozyme and T7 terminator. T7 RNA polymerase transcription of pPH3 would produce 1261-nt long negative-sense RNA consisting of the 155-nt trailer at the 5' end, 75-nt of the trailer's complement at the 3' end and 1031-nt L gene end in the middle. After autolytic cleavage, the 3' end of the panhandle-type RNA analog should be exactly complementary to the authentic 5' end of the genome.

To examine the ability of these two constructs to generate transcripts of the appropriate length in 293 cells, pWT1 and pPH3 were transfected in to vTF7-3 infected cells, respectively, and RNAs were labeled with [$^3$H]uridine for 8 hours at 16 hours posttransfection. The total cytoplasmic RNA species synthesized during this period was analyzed by electrophoresis on agarose-urea gel. A major species of labeled RNA of 1.6 Kb from the pWT1 transfected sample and 1.2 Kb from the pPH3 transfected sample was observed, but not in vTF7-3 infected only and the uninfected cells. The minor bands migrating slightly slower than the major negative-sense RNA transcripts were RNA that had not undergone the autolytic cleavage by the time of analysis. The identity of these cleaved

and uncleaved transcripts was confirmed later by comparison with the cleaved and uncleaved transcripts from the same plasmids generated by *in vitro* transcription. More than 90% of the transcripts synthesized during the labeling period was cleaved by the ribozyme, releasing a 200 base RNA that contained the ribozyme and terminator sequences and that migrated near the bottom of the gel.

*Encapsidation and replication of genomic RNA analogs*

The active template for RNA synthesis by negative-strand RNA viruses is the RNA in the form of a ribonucleocapsid. To determine whether the RNAs transcribed in cells by T7 polymerase could be encapsidated with the nucleocapsid protein and replicated, vTF7-3-infected cells were transfected with pWT1 or pPH3 and combinations of plasmids encoding the N, P and L proteins. At 16 hours posttransfection, the cells were exposed to [$^3$H]uridine for 6 hours. Encapsidated and replicated RNAs were selected by immunoprecipitation and analyzed on an agarose-urea gel. Immunoprecipitation of [$^3$H] labeled RNA by anti RS virus polyclonal serum demonstrated that encapsidation of WT and PH type RNA analogs occurred when pRSV-N, pRSV-P and pRSV-L were cotransfected. However, in the absence of pRSV-L, encapsidated RNA was barely detected. These results suggested that only a small percentage of the original T7 negative-sense RNA transcripts was encapsidated and that the majority of the encapsidated RNA arose from replication of the original transcripts by the RS virus polymerase. To test whether the labeled and encapsidated RNA was replicated by the RS virus RNA dependent RNA polymerase, the effect of actinomycin D on synthesis and encapsidation of RNA was analyzed. Actinomycin D inhibits DNA dependent RNA synthesis, but not RNA dependent RNA synthesis.

In the presence of actinomycin D, incorporation of [$^3$H ] uridine into RS virus genomic analog was completely blocked when only pRSV-N and pRSV-P were present in the cotransfection. However, when pRSV-L was included in the cotransfection, synthesis of the genomic analog was resistant to the drug and readily detected. The results demonstrated that the RNAs were indeed the products of replication by the RS virus polymerase. The majority of encapsidated RNAs represent the replicated RNAs However, the amount of RNA replicated from the wild-type genomic analog is much less than that from the panhandle one, although a similar molar ratio of plasmids was used in the transfection. Due to its higher RNA replication efficiency, the panhandle type analog pPH3 was used as a model to determine the *trans*-acting protein requirements for RNA replication and to detect the strand-specific RNA synthesis.

*The N, P and L proteins are the minimal trans-acting protein requirements for RNA replication*

To determine the minimal *trans*-acting protein requirements for RS virus genomic RNA replication, and to optimize the conditions of RNA replication, pPH3 transfected-cells were cotransfected with various combinations of plasmids encoding the N, P and L proteins. At 16 hours posttransfection, the cells were labeled with [$^3$H]uridine in the presence of actinomycin D for 6 hours. The RNAs extracted from cell lysates were analyzed by electrophoresis on an agarose-urea gel. The results clearly showed that any combination of two of these three plasmids in the cotransfection did not support RNA replication. Only when all three plasmids were present in the cotransfection did replication of the panhandle-type RNA analog occur. This clearly defined that the N, P and L proteins were the minimal *trans*-acting protein requirements for RNA replication of the RS virus genomic analog. As the amount of pRSV-L was increased from 0.25 ug -1 ug in the cotransfection, the yield of replicated RNA products also increased. However, when 2 ug of pRSV-L was cotransfected, the efficiency of replication no longer increased. Maximum RNA replication occurred when the molar ratio of transfected N, P and L genes was 12:5:1.

To test the specificity of the requirement for the viral RNA dependent RNA polymerase for RS virus RNA replication, a VSV L gene plasmid that had been shown to support VSV RNA replication in a similar system (Pattnaik , A.K. et. al., (1992) Infectious defective interfering particles of VSV from transcripts of a cDNA clone. *Cell 69*:1011-1020; Wertz, G. et al., (1994) *Proc. Natl. Acad. Sci. USA*,, 91, 8587-8591) and a truncated form of RS virus L gene plasmid that expressed a 170 kDa polypeptide were used to replace pRSV-L in the cotransfection. Neither the heterologous VSV RNA polymerase nor the truncated RS virus polymerase supported RNA replication. These data demonstrate that the RNA replication of the genomic analog indeed requires RS virus specific and functional polymerase.

*RNase protection assay demonstrates the synthesis of positive-strand RNA*

During RNA replication of negative-stranded RNA viruses, the encapsidated negative sense genome must first replicate a positive sense RNA antigenome, which in turn would be encapsidated and serve as a template for the synthesis of progeny negative-sense RNA. Therefore, the synthesis of a positive-strand intermediate is critical evidence for establishing that replication of the original negative-strand RNA has occurred. To test RS virus positive-strand RNA synthesis, an RNase protection assay was carried out with a strand-specific probe. Encapsidated RNA was selected by immunoprecipitation from cells

cotransfected with pPH3 and combinations of the N, P and L gene plasmids. A 391-nt long, [$^{35}$S]-labeled RNA probe was used, of which 360 nucleotides were transcribed from the L gene sequences and complementary to the positive-sense RNA, and the other 31 nucleotides corresponded to the polylinker region of the vector. Hybridization of the probe with the positive-strand RNA should produce a double strand RNA hybrid which, after nuclease digestion to remove the overhanging nucleotides, would be 360 base pairs long.

Indeed, electrophoretic analysis of the protected RNA products demonstrated that the positive-strand RNA was synthesized when all three viral N, P and L gene plasmids were cotransfected, but not in the absence of the L gene plasmid in the cotransfection. This protected RNA product migrated at the predicted size (360 nt). The undigested probe (391 nt) hybridized with yeast RNA and was completely degraded following treatment with RNase, thus indicating that the nuclease digestion was complete. These data demonstrated that positive-strand RNA was replicated from the initial negative sense RNA transcribed in cells, and confirmed that RNA replication occurred only when all three viral proteins, N, P and L were provided by cotransfection. The presence of positive-sense RNA was also confirmed by primer extension analysis with a negative-sense oligonucleotide primer.

## Example 2 Recovery of Infectious VSV Entirely from cDNA Clones

### Materials and Methods

*Plasmid construction and transfections*

A full length cDNA clone of VSV was assembled from clones of each of the VSV genes and intergenic junctions, using standard cloning techniques (Sambrook, J. et al. (1989) *Molecular Cloning: A Laboratory Manual,* 2d ed. Cold Spring Harbor Laboratory Press, New York). Whenever possible, clones that were known to encode functional VSV proteins were used for the construction. These clones were assembled into a full length cDNA and inserted in both orientations between the bacteriophage T7 promoter and a cDNA copy of the self cleaving riobzyme from the antigenomic strand of HDV. The resulting plasmids were named pVSV1(+) and pVSV1(-) to reflect the polarity of the T7 transcript they generated: VSV anti-genomic or genomic RNA respectively. The T7 transcripts contained two non-VSV nucleotides (GG) at their 5' end but were cleaved by the HDV riobzyme to generate a 3' terminus which corresponded precisely to the 3' end of the VSV antigenomic (Figure 5) or genomic sequence.

Transfection of plasmids into BHK1 cells infected with vTF7-3 was performed using the conditions and quantities of support plasmids described previously (Pattnaik, A.K. et al. (1992) *Cell* **69**, 1011-1020), and up to 5μg of the plasmids pVSV1(+) or pVSV1(-). Transfected cells were incubated at either 31°C or 37°C. For some experiments, the pVSV1(+) and pVSV1(-) plasmids were linearized by digestion at a unique NheI site located downstream of the T7 terminator in the pGEM3 based plasmids. RNAs made by run-off transcription from these linearized DNAs still contained the HDV ribozyme whose production of a perfect 3' end by self-cleavage is essential for VSV RNA replication (Pattnaik, A.K. et al. (1992) *Cell* **69**, 1011-1020). All experiments involving pVSV1(+) were performed in a newly constructed building, in a laboratory in which wild-type VSV had never been used.

To identify cDNA-derived virus unambiguously, several genetic markers were incorporated into the full length cDNA clones. All 5 genes were of the Indiana serotype of VSV, but whereas the N, P, M and L genes originated from the San Juan strain, the G gene (kindly provided by Elliot Lefkowitz) was from the a Orsay strain. In addition the functional P clone has 28 nucleotide sequences differences from the published San Juan sequence and in the case of pVSV1(+) the 516 nucleotides at the 5' end of the VSV genome originated from pDI, the clone of DI-T RNA (Pattnaik, A.K. et al. (1992) *Cell* **69**, 1011-1020). This region contained several nucleotide differences from the published VSV Indiana San Juan sequence. Specifically the nucleotide differences G11038A, A11070C, and an insertion of an A residue at nucleotide 11140 were used to unambiguously distinguish cDNA-derived virus.

To examine the behavior of T7 RNA polymerase at a VSV intergenic junction, a BglII fragment that encompassed the NP intergenic junction of VSV (nucleotides 1236-1685) was inserted in both orientations into the unique BglII site of plasmid 8 (Wertz, G. W. et al (1994) *Proc. Natl. Acad. Sci. USA* **91**, 8587-8591). This plasmid contained 210 nucleotides form the 3' end of VSV RNA and 265 nucleotides from the 5' end, joined at a unique BglII site and placed between the T7 promoter and the HDV ribozyme. A positive-sense version of plasmid 8 was generated and used to accommodate the NP intergenic junction sequence in an identical manner. These plasmids were named 8(-) and 8(+) to reflect the polarity of the T7 transcript they generated, with an additional suffix to indicate whether the NP intergenic junction was in the natural (NP) or inverted (PN) orientation with respect to the surrounding VSV sequences (see Figure 5).

*Virus production and neutralization*

The medium from transfected cells was harvested at 15 to 48 hours post transfection, clarified by centrifugation (14 000 x g for 5 minutes), and virus titers were monitored by plaque assay on BSC40 cells in the presence of 25μg per ml cytosine arabinoside (ara-c), to inhibit replication of VV. Neutralization assays of virus were performed by incubation with a mouse polyclonal serum raised against purified VSV, by incubation with antiserum for 30 minutes at room temperature in DMEM. This approach also allowed titration of VV, by plaque assay in the absence of ara-c.

*Virus characterization*

Virus amplification, radioactive labeling of RNA with [$^3$H]uridine (33μCi per ml; 1 Ci = 37 GBq) and proteins with [$^{35}$S] methionine (10μCi per ml) or [$^3$H]leucine (50μ Ci per ml) and their electrophoretic analyses were performed as described previously (Pattnaik, A.K. et al. (1990) *J. Virol.* **64,** 2948-2957). Viral RNA was purified from $10^8$ pfu of amplified cDNA-derived VSV and reverse transcribed using AMV reverse transcriptase (Life Sciences) and a primer that annealed to negative-sense RNA at nucleotides 11026-11043 of the VSV genome. Approximately 1/10th of this reaction was used for DNA amplification by PCR. PCR reactions were carried out using the primer described above and a second primer that annealed to the extreme 3' end of positive-sense VSV RNA (nucleotides 11161-11144). PCR products were cloned and sequenced using standard techniques (Sambrook, J. et al. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, New York).

*In vitro transcription*

RNA was generated *in vitro* using T7 RNA polymerase (GIBCO-BRL) according to manufacturer's instructions, except that rNTP concentrations were elevated to 2.5mM, and supplemented with [$^3$H] UTP (80μCi per ml). The RNA products of *in vitro* transcription were resolved by electrophoresis on 1% agarose-urea gels and visualized by fluorography. It should be noted that RNA mobility in the pH 3.0 agarose-urea gels is a function of base composition as well as size (Lerach, H., (1977) *Biochemistry*, **16**, 4743-4751).

**Results**

*Construction of a full length cDNA clone of VSV and recovery of infectious virus*

A full-length cDNA of the RNA genome of VSV was assembled from clones of each of the five VSV genes and their intergenic regions and inserted into a pGEM3 based transcription plasmid between the T7 promoter and the HDV ribozyme (Figure 6). Plasmids containing the cDNA in both orientations were constructed and designated pVSV1(+) and pVSV1(-) to reflect the polarity of the T7 transcript they generated: VSV anti-genomic or genomic RNA respectively. pVSV1(+) was transfected into BHK21 cells that expressed T7 RNA polymerase from a VV recombinant, together with T7 transcription plasmids that separately encoded the VSV N, P, and L proteins. These latter three "support" plasmids provided sources of the VSV proteins necessary to support encapsidation of the primary naked transcript, and to provide a source of polymerase for replication and transcription of this RNA. Control transfections included cells that received pVSV1(+) but no support plasmids, and cells that received the support plasmids but no pVSV1(+). After incubation at 31°or 37°C, the culture media were harvested, diluted and monitored by plaque assay for the presence of infectious VSV. Infectious virus was recovered reproducibly from cells that received both pVSV1(+) and the N, P, and L support plasmids, but not in either of the two control transfections in which either pVSV1(+) or the VSV support plasmids were omitted (Table 2). The efficiency of recovery varied among different experiments, and was affected by the time of harvest, the temperature of incubation of the cells, and whether the genomic cDNA plasmid was linearized before transfection. Among the conditions tested, the highest level of recovery was $8 \times 10^4$ pfu per ml in the 1.5 ml of medium from $10^6$ cells that had received 5µg of linearized pVSV1(+) and been incubated at 31°C for 45 hours.

Table 2

Recovery of Infectious Virus from pVSV1(+)

| VSV Plasmids Transfected | | Virus Yield | Virus Recovered in |
|---|---|---|---|
| Genomic | Support | (pfu/ml) | X/Y transfections |
| pVSV1(+) | ----- | < 10 | 0/8 |
| ----- | N, P, L | < 10 | 0/4 |
| pVSV1(+) | N, P, L | < 10 to $8 \times 10^4$ | 9/12(a) |
| pVSV1(+) | N, P, L, M, G | < 10 to $1 \times 10^2$ | 1/6 |
| pVSV1(-) | N, P, L | < 10 | 0/27 |
| pVSV1(-) | N, P, L, M, G | < 10 | 0/12 |

The only source of the VSV G and M proteins in this experiment was via VSV-mediated transcription of the replicated genomic RNA. Indeed, when support plasmids that expressed the M and G proteins were included in the transfection mixture together with those that expressed N, P and L, under conditions that support the production of infectious VSV DI particles from a DI cDNA clone (Pattnaik, A.K. et al. (1992) *Cell* **69**, 1011-1020), the recovery of infectious virus was strongly suppressed (Table 2), perhaps because VSV M protein can inhibit both viral and cellular transcription (Clinton, G.M. et al. (1978) *Cell* **15**, 1455-1462; Black, B.L. et al. (1992) *J. Virol.* **66**, 4058-4064).

Plasmid pVSV(-) which was designed to express a full-length negative-sense copy of the VSV genome, failed repeatedly to yield infectious virus, either when supported by the expression of N, P, and L proteins, or by the full complement of five VSV proteins (Table 1).

*Neutralization of Recovered Virus by Anti-VSV Antiserum*

The virus that yielded the plaques was identified as VSV because plaque-formation was completely inhibited by a mouse polyclonal anti-serum raised against purified wild-type VSV. However, since the transfected cells had been infected with the VV recombinant vTF7-3 to provide T7 RNA polymerase, the harvested culture medium also contained infectious VV. Plaque assays performed in the presence of anti-VSV anti-serum (and in the absence of ara-c) showed that under all conditions of transfection, VV titers of 1-2 x $10^6$ pfu per ml were released from the infected transfected cells. However, the VV plaques were less than one tenth the size of the VSV plaques, easily distinguished from them, and completely suppressed by ara-c which inhibits VV DNA replication.

*RNA and protein synthesis activities of recovered VSV*

To provide further evidence that the virus recovered from transfections of pVSV1(+) was VSV, the RNAs and proteins synthesized by this virus were compared with those made by authentic VSV. For RNA analysis the supernatant fluids harvested from primary transfections were amplified once in BHK21 cells in the presence of ara-c. The resultant supernatants were used to infect BHK21 cells which were exposed to [$^3$H]uridine in the presence of actinomycin-D from 3 to 6 hours post infection. Cytoplasmic extracts were prepared, RNAs were harvested, resolved by electrophoresis on 1.75% agarose-urea gels, and detected by fluorography. RNAs that comigrated with authentic VSV genomic RNA and the five mRNAs were synthesized following infection with samples harvested from transfections

that received pVSV1(+) and three support plasmids, N, P and L. No VSV RNAs were detected following passage of supernatants from transfections that did not receive both pVSV1(+) and the support plasmids.

Viral protein synthesis was monitored following the infection of BHK21 cells at an MOI of 5. Cells were starved for methionine for 30 minutes prior to incorporation of [$^{35}$S]methionine from 1 hour post-infection for 5 hours. Cytoplasmic extracts were prepared and proteins were analyzed on a 10% polyacrylamide-SDS gel. Virus recovered from transfections of pVSV1(+) displayed a protein profile that closely resembled those of the San Juan and Orsay strains of VSV Indiana. Furthermore the proteins that were specifically immunoprecipitated by a VSV specific antiserum (which reacts poorly with the VSV M and P proteins) were similar in the three cases, providing further evidence that the recovered virus was VSV. However there were minor differences in the mobility of the proteins from the recovered virus, M protein providing the clearest example. These different mobilities were characteristic of the proteins encoded by the support plasmids that had been used to construct pVSV1(+), and thus provided evidence that the genome of the recovered virus was derived from the cDNA clone.

*VSV recovered from the cDNA clone contained characteristic sequence markers*

During the construction of pVSV1(+) several nucleotide sequence markers were introduced with the 5' terminal 516 nucleotides which originated from the cDNA clone of DI-T RNA (Pattnaik, A.K. et al. (1992) *Cell* **69**, 1011-1020). To examine the nucleotide sequence of the 5' end of the genome of the recovered virus; RT-PCR was performed. RNA was purified from the recovered virus after 3 passages, and the region from nucleotide 11026 to the extreme 5' end of the genome (nucleotide 11161) was amplified, cloned and sequenced. In comparison to the published Indiana San Juan virus sequence the following nucleotide differences were noted; nucleotides G11038A, A11070C, and an insertion of an A residue at nucleotide 11140. These results revealed that the nucleotide sequence of this region of the genome of the recovered virus was identical to the cDNA clone, and hence that the recovered virus originated from pVSV1(+).

*Genome length negative-sense RNA transcripts of VSV were not synthesized efficiently by bacteriophage T7 RNA polymerase.*

In marked contrast to our success in recovering infectious VSV from pVSV1(+), attempts to generate infectious virus from negative-sense RNA transcripts were uniformly unsuccessful (Table 1). This was surprising, because success with negative-sense

T7 transcripts of DI-T RNA (Pattnaik, A.K. et al. (1992) *Cell* **69**, 1011-1020; Pattnaik, A.K. et al. (1995) *Virology* **206**, 760-764) and with several subgenomic replicons (Wertz, G.W. et al. (1994) *Proc. Natl. Acad. Scie. USA* **91**, 8587-8591) had suggested no inherent problems with this strategy. We therefore compared the ability of T7 RNA polymerase to synthesize genome length positive and negative-sense transcripts of VSV *in vitro*. pVSV(+) and pVSV1(-) were linearized at the unique Nhe1 site and transcribed *in vitro* by T7 RNA polymerase in the presence of [$^3$H]UTP. The products were analyzed on 1% agarose-urea gels, and detected by fluorography. Whereas transcripts of pVSV1(+) were predominantly genome length, the majority of T7 transcripts of pVSV1(-) were clearly smaller than the VSV RNA. Clearly this apparent inability of T7 RNA polymerase to synthesize full length negative-sense transcripts of VSV RNA could explain the lack of infectivity of pVSV1(-).

The natural signal for transcriptional termination by T7 RNA polymerase is a strong hairpin structure followed by 6 U residues in the nascent RNA (Rosenberg, A.H. et al. (1987) *Gene* **56**, 125-135). A run of 7 U residues exists at each of the intergenic junctions in the negative-strand of VSV RNA, and among the transcription products from pVSV1(-) were four discrete RNAs of the appropriate size to represent the products of termination at the intergenic junctions. The behavior of T7 RNA polymerase when transcribing a VSV intergenic junction in the negative-sense, as compared with the positive-sense was investigated. The NP intergenic region was cloned in both the positive and negative orientation into transcription plasmids between the T7 promoter and the HDV ribozyme/T7 terminator cassettes. *In vitro* transcriptions were performed on each of these circular plasmids in the presence of [$^3$H]UTP and the RNA products were analyzed on a 1% agarose-urea gel and detected by fluorography. Plasmid 8(+)NP, which generated positive-sense transcripts of the NP intergenic junction, gave the expected two RNAs that resulted form transcriptional termination at the T7 termination signal and the subsequent ribozyme mediated self cleavage to generate authentic VSV 3' termini. The smaller (200 nucleotides) product of self cleavage had run off this gel. In contrast the two plasmids 8(-)NP and 8(+)PN, that were designed to generate negative-sense transcripts of the NP intergenic junction, each yielded a major smaller RNA product in addition to the expected products of T7 termination and self-cleavage. The sizes of these smaller RNAs were consistent with termination at or very close to the NP intergenic junction, as shown by comparison with the size of the RNA made by run-off transcription from plasmid 8(+)PN linearized at the EcoRV site which is 7 nucleotides from the NP intergenic junction. These analyses show that T7 RNA polymerase terminated near the VSV NP intergenic junction when synthesizing a negative-sense RNA, but not when generating a positive-sense RNA transcript. Furthermore, the RNA products directed by pVSV1(-) suggest that similar termination occurred to a greater or lesser extent at the other intergenic junctions. The cumulative effect of this

incomplete transcriptional termination by T7 RNA polymerase, at each intergenic junction in the negative-sense transcript, probably accounts for the difference in the RNAs transcribed *in vitro* from pVSV1(-) and pVSV1(+).

## Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Dr. Gail W. Wertz et al.

(ii) TITLE OF INVENTION: Gene Therapy Vectors and Vaccines Based on Non-Segmented Negative Stranded RNA Viruses

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/316,438
(B) FILING DATE: 30-SEP-1994

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Arnold, Beth E. (BEA)
(B) REGISTRATION NUMBER: 35,430
(C) REFERENCE/DOCKET NUMBER: UAG-010CP

(ix) TELECOMMUNICATION INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6578 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 9..6504

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GGGACAAA ATG GAT CCC ATT ATT AAT GGA AAT TCT GCT AAT GTT TAT CTA          50
         Met Asp Pro Ile Ile Asn Gly Asn Ser Ala Asn Val Tyr Leu
           1               5                  10

ACC GAT GGT TAT TTA AAA GGT GTT ATC TCT TTC TCA GAG TGT AAT GCT          98
Thr Asp Gly Tyr Leu Lys Gly Val Ile Ser Phe Ser Glu Cys Asn Ala
 15                  20                  25                  30

TTA GGA AGT TAC ATA TTC AAT GGT CCT TAT CTC AAA AAT GAT TAT ACC         146
Leu Gly Ser Tyr Ile Phe Asn Gly Pro Tyr Leu Lys Asn Asp Tyr Thr
                    35                  40                  45

AAC TTA ATT AGT AGA CAA AAT CCA TTA ATA GAA CAC ATG AAT CTA AAG         194
Asn Leu Ile Ser Arg Gln Asn Pro Leu Ile Glu His Met Asn Leu Lys
                50                  55                  60

AAA CTA AAT ATA ACA CAG TCC TTA ATA TCT AAG TAT CAT AAA GGT GAA         242
Lys Leu Asn Ile Thr Gln Ser Leu Ile Ser Lys Tyr His Lys Gly Glu
            65                  70                  75

ATA AAA TTA GAA GAA CCT ACT TAT TTT CAG TCA TTA CTT ATG ACA TAC         290
Ile Lys Leu Glu Glu Pro Thr Tyr Phe Gln Ser Leu Leu Met Thr Tyr
        80                  85                  90

AAG AGT ATG ACC TCG TCA GAA CAG ATT GCT ACC ACT AAT TTA CTT AAA         338
Lys Ser Met Thr Ser Ser Glu Gln Ile Ala Thr Thr Asn Leu Leu Lys
 95                  100                 105                 110

AAG ATA ATA AGA AGA GCT ATA GAA ATA AGT GAT GTC AAA GTC TAT GCT         386
Lys Ile Ile Arg Arg Ala Ile Glu Ile Ser Asp Val Lys Val Tyr Ala
                115                 120                 125

ATA TTG AAT AAA CTA GGG CTT AAA GAA AAG GAC AAG ATT AAA TCC AAC         434
Ile Leu Asn Lys Leu Gly Leu Lys Glu Lys Asp Lys Ile Lys Ser Asn
            130                 135                 140

AAT GGA CAA GAT GAA GAC AAC TCA GTT ATT ACG ACC ATA ATC AAA GAT         482
Asn Gly Gln Asp Glu Asp Asn Ser Val Ile Thr Thr Ile Ile Lys Asp
        145                 150                 155

GAT ATA CTT TCA GCT GTT AAA GAT AAT CAA TCT CAT CTT AAA GCA GAC         530
Asp Ile Leu Ser Ala Val Lys Asp Asn Gln Ser His Leu Lys Ala Asp
    160                 165                 170

AAA AAT CAC TCT ACA AAA CAA AAA GAC ACA ATC AAA ACA ACA CTC TTG         578
Lys Asn His Ser Thr Lys Gln Lys Asp Thr Ile Lys Thr Thr Leu Leu
175                 180                 185                 190

AAG AAA TTG ATG TGT TCA ATG CAA CAT CCT CCA TCA TGG TTA ATA CAT         626
Lys Lys Leu Met Cys Ser Met Gln His Pro Pro Ser Trp Leu Ile His
                195                 200                 205
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TGG TTT AAC TTA TAC ACA AAA TTA AAC AAC ATA TTA ACA CAG TAT CGA          674
Trp Phe Asn Leu Tyr Thr Lys Leu Asn Asn Ile Leu Thr Gln Tyr Arg
            210                 215                 220

TCA AAT GAG GTA AAA AAC CAT GGG TTT ACA TTG ATA GAT AAT CAA ACT          722
Ser Asn Glu Val Lys Asn His Gly Phe Thr Leu Ile Asp Asn Gln Thr
        225                 230                 235

CTT AGT GGA TTT CAA TTT ATT TTG AAC CAA TAT GGT TGT ATA GTT TAT          770
Leu Ser Gly Phe Gln Phe Ile Leu Asn Gln Tyr Gly Cys Ile Val Tyr
    240                 245                 250

CAT AAG GAA CTC AAA AGA ATT ACT GTG ACA ACC TAT AAT CAA TTC TTG          818
His Lys Glu Leu Lys Arg Ile Thr Val Thr Thr Tyr Asn Gln Phe Leu
255                 260                 265                 270

ACA TGG AAA GAT ATT AGC CTT AGT AGA TTA AAT GTT TGT TTA ATT ACA          866
Thr Trp Lys Asp Ile Ser Leu Ser Arg Leu Asn Val Cys Leu Ile Thr
                275                 280                 285

TGG ATT AGT AAC TGC TTG AAC ACA TTA AAT AAA AGC TTA GGC TTA AGA          914
Trp Ile Ser Asn Cys Leu Asn Thr Leu Asn Lys Ser Leu Gly Leu Arg
            290                 295                 300

TGC GGA TTC AAT AAT GTT ATC TTG ACA CAA CTA TTC CTT TAT GGA GAT          962
Cys Gly Phe Asn Asn Val Ile Leu Thr Gln Leu Phe Leu Tyr Gly Asp
        305                 310                 315

TGT ATA CTA AAG CTA TTT CAC AAT GAG GGG TTC TAC ATA ATA AAA GAG         1010
Cys Ile Leu Lys Leu Phe His Asn Glu Gly Phe Tyr Ile Ile Lys Glu
    320                 325                 330

GTA GAG GGA TTT ATT ATG TCT CTA ATT TTA AAT ATA ACA GAA GAA GAT         1058
Val Glu Gly Phe Ile Met Ser Leu Ile Leu Asn Ile Thr Glu Glu Asp
335                 340                 345                 350

CAA TTC AGA AAA CGA TTT TAT AAT AGT ATG CTC AAC AAC ATC ACA GAT         1106
Gln Phe Arg Lys Arg Phe Tyr Asn Ser Met Leu Asn Asn Ile Thr Asp
                355                 360                 365

GCT GCT AAT AAA GCT CAG AAA AAT CTG CTA TCA AGA GTA TGT CAT ACA         1154
Ala Ala Asn Lys Ala Gln Lys Asn Leu Leu Ser Arg Val Cys His Thr
            370                 375                 380

TTA TTA GAT AAG ACA GTG TCC GAT AAT ATA ATA AAT GGC AGA TGG ATA         1202
Leu Leu Asp Lys Thr Val Ser Asp Asn Ile Ile Asn Gly Arg Trp Ile
        385                 390                 395

ATT CTA TTA AGT AAG TTC CTT AAA TTA ATT AAG CTT GCA GGT GAC AAT         1250
Ile Leu Leu Ser Lys Phe Leu Lys Leu Ile Lys Leu Ala Gly Asp Asn
    400                 405                 410

AAC CTT AAC AAT CTG AGT GAA CTA TAT TTT TTG TTC AGA ATA TTT GGA         1298
Asn Leu Asn Asn Leu Ser Glu Leu Tyr Phe Leu Phe Arg Ile Phe Gly
415                 420                 425                 430
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CAC CCA ATG GTA GAT GAA AGA CAA GCC ATG GAT GCT GTT AAA ATT AAT      1346
His Pro Met Val Asp Glu Arg Gln Ala Met Asp Ala Val Lys Ile Asn
                435                 440                 445

TGC AAT GAG ACC AAA TTT TAC TTG TTA AGC AGT CTG AGT ATG TTA AGA      1394
Cys Asn Glu Thr Lys Phe Tyr Leu Leu Ser Ser Leu Ser Met Leu Arg
            450                 455                 460

GGT GCC TTT ATA TAT AGA ATT ATA AAA GGG TTT GTA AAT AAT TAC AAC      1442
Gly Ala Phe Ile Tyr Arg Ile Ile Lys Gly Phe Val Asn Asn Tyr Asn
        465                 470                 475

AGA TGG CCT ACT TTA AGA AAT GCT ATT GTT TTA CCC TTA AGA TGG TTA      1490
Arg Trp Pro Thr Leu Arg Asn Ala Ile Val Leu Pro Leu Arg Trp Leu
    480                 485                 490

ACT TAC TAT AAA CTA AAC ACT TAT CCT TCT TTG TTG GAA CTT ACA GAA      1538
Thr Tyr Tyr Lys Leu Asn Thr Tyr Pro Ser Leu Leu Glu Leu Thr Glu
495                 500                 505                 510

AGA GAT TTG ATT GTG TTA TCA GGA CTA CGT TTC TAT CGT GAG TTT CGG      1586
Arg Asp Leu Ile Val Leu Ser Gly Leu Arg Phe Tyr Arg Glu Phe Arg
                515                 520                 525

TTG CCT AAA AAA GTG GAT CTT GAA ATG ATT ATA AAT GAT AAA GCT ATA      1634
Leu Pro Lys Lys Val Asp Leu Glu Met Ile Ile Asn Asp Lys Ala Ile
            530                 535                 540

TCA CCT CCT AAA AAT TTG ATA TGG ACT AGT TTC CCT AGA AAT TAC ATG      1682
Ser Pro Pro Lys Asn Leu Ile Trp Thr Ser Phe Pro Arg Asn Tyr Met
        545                 550                 555

CCA TCA CAC ATA CAA AAC TAT ATA GAA CAT GAA AAA TTA AAA TTT TCC      1730
Pro Ser His Ile Gln Asn Tyr Ile Glu His Glu Lys Leu Lys Phe Ser
    560                 565                 570

GAG AGT GAT AAA TCA AGA AGA GTA TTA GAG TAT TAT TTA AGA GAT AAC      1778
Glu Ser Asp Lys Ser Arg Arg Val Leu Glu Tyr Tyr Leu Arg Asp Asn
575                 580                 585                 590

AAA TTC AAT GAA TGT GAT TTA TAC AAC TGT GTA GTT AAT CAA AGT TAT      1826
Lys Phe Asn Glu Cys Asp Leu Tyr Asn Cys Val Val Asn Gln Ser Tyr
                595                 600                 605

CTC AAC AAC CCT AAT CAT GTG GTA TCA TTG ACA GGC AAA GAA AGA GAA      1874
Leu Asn Asn Pro Asn His Val Val Ser Leu Thr Gly Lys Glu Arg Glu
            610                 615                 620

CTC AGT GTA GGT AGA ATG TTT GCA ATG CAA CCG GGA ATG TTC AGA CAG      1922
Leu Ser Val Gly Arg Met Phe Ala Met Gln Pro Gly Met Phe Arg Gln
        625                 630                 635

GTT CAA ATA TTG GCA GAG AAA ATG ATA GCT GAA AAC ATT TTA CAA TTC      1970
Val Gln Ile Leu Ala Glu Lys Met Ile Ala Glu Asn Ile Leu Gln Phe
    640                 645                 650

TTT CCT GAA AGT CTT ACA AGA TAT GGT GAT CTA GAA CTA CAA AAA ATA      2018
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Phe Pro Glu Ser Leu Thr Arg Tyr Gly Asp Leu Glu Leu Gln Lys Ile
655                 660                 665                 670

TTA GAA TTG AAA GCA GGA ATA AGT AAC AAA TCA AAT CGC TAC AAT GAT      2066
Leu Glu Leu Lys Ala Gly Ile Ser Asn Lys Ser Asn Arg Tyr Asn Asp
                675                 680                 685

AAT TAC AAC AAT TAC ATT AGT AAG TGC TCT ATC ATC ACA GAT CTC AGC      2114
Asn Tyr Asn Asn Tyr Ile Ser Lys Cys Ser Ile Ile Thr Asp Leu Ser
            690                 695                 700

AAA TTC AAT CAA GCA TTT CGA TAT GAA ACG TCA TGT ATT TGT AGT GAT      2162
Lys Phe Asn Gln Ala Phe Arg Tyr Glu Thr Ser Cys Ile Cys Ser Asp
        705                 710                 715

GTG CTG GAT GAA CTG CAT GGT GTA CAA TCT CTA TTT TCC TGG TTA CAT      2210
Val Leu Asp Glu Leu His Gly Val Gln Ser Leu Phe Ser Trp Leu His
    720                 725                 730

TTA ACT ATT CCT CAT GTC ACA ATA ATA TGC ACA TAT AGG CAT GCA CCC      2258
Leu Thr Ile Pro His Val Thr Ile Ile Cys Thr Tyr Arg His Ala Pro
735                 740                 745                 750

CCC TAT ATA GGA GAT CAT ATT GTA GAT CTT AAC AAT GTA GAT GAA CAA      2306
Pro Tyr Ile Gly Asp His Ile Val Asp Leu Asn Asn Val Asp Glu Gln
                755                 760                 765

AGT GGA TTA TAT AGA TAT CAC ATG GGT GGC ATC GAA GGG TGG TGT CAA      2354
Ser Gly Leu Tyr Arg Tyr His Met Gly Gly Ile Glu Gly Trp Cys Gln
            770                 775                 780

AAA CTA TGG ACC ATA GAA GCT ATA TCA CTA TTG GAT CTA ATA TCT CTC      2402
Lys Leu Trp Thr Ile Glu Ala Ile Ser Leu Leu Asp Leu Ile Ser Leu
        785                 790                 795

AAA GGG AAA TTC TCA ATT ACT GCT TTA ATT AAT GGT GAC AAT CAA TCA      2450
Lys Gly Lys Phe Ser Ile Thr Ala Leu Ile Asn Gly Asp Asn Gln Ser
    800                 805                 810

ATA GAT ATA AGC AAA CCA ATC AGA CTC ATG GAA GGT CAA ACT CAT GCT      2498
Ile Asp Ile Ser Lys Pro Ile Arg Leu Met Glu Gly Gln Thr His Ala
815                 820                 825                 830

CAA GCA GAT TAT TTG CTA GCA TTA AAT AGC CTT AAA TTA CTG TAT AAA      2546
Gln Ala Asp Tyr Leu Leu Ala Leu Asn Ser Leu Lys Leu Leu Tyr Lys
                835                 840                 845

GAG TAT GCA GGC ATA GGC CAC AAA TTA AAA GGA ACT GAG ACT TAT ATA      2594
Glu Tyr Ala Gly Ile Gly His Lys Leu Lys Gly Thr Glu Thr Tyr Ile
            850                 855                 860

TCA CGA GAT ATG CAA TTT ATG AGT AAA ACA ATT CAA CAT AAC GGT GTA      2642
Ser Arg Asp Met Gln Phe Met Ser Lys Thr Ile Gln His Asn Gly Val
        865                 870                 875
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TAT TAC CCA GCT AGT ATA AAG AAA GTC CTA AGA GTG GGA CCG TGG ATA      2690
Tyr Tyr Pro Ala Ser Ile Lys Lys Val Leu Arg Val Gly Pro Trp Ile
    880                 885                 890

AAC ACT ATA CTT GAT GAT TTC AAA GTG AGT CTA GAA TCT ATA GGT AGT      2738
Asn Thr Ile Leu Asp Asp Phe Lys Val Ser Leu Glu Ser Ile Gly Ser
895                 900                 905                 910

TTG ACA CAA GAA TTA GAA TAT AGA GGT GAA AGT CTA TTA TGC AGT TTA      2786
Leu Thr Gln Glu Leu Glu Tyr Arg Gly Glu Ser Leu Leu Cys Ser Leu
                915                 920                 925

ATA TTT AGA AAT GTA TGG TTA TAT AAT CAG ATT GCT CTA CAA TTA AAA      2834
Ile Phe Arg Asn Val Trp Leu Tyr Asn Gln Ile Ala Leu Gln Leu Lys
            930                 935                 940

AAT CAT GCA TTA TGT AAC AAT AAA CTA TAT TTG GAC ATA TTA AAG GTT      2882
Asn His Ala Leu Cys Asn Asn Lys Leu Tyr Leu Asp Ile Leu Lys Val
        945                 950                 955

CTG AAA CAC TTA AAA ACC TTT TTT AAT CTT GAT AAT ATT GAT ACA GCA      2930
Leu Lys His Leu Lys Thr Phe Phe Asn Leu Asp Asn Ile Asp Thr Ala
    960                 965                 970

TTA ACA TTG TAT ATG AAT TTA CCC ATG TTA TTT GGT GGT GGT GAT CCC      2978
Leu Thr Leu Tyr Met Asn Leu Pro Met Leu Phe Gly Gly Gly Asp Pro
975                 980                 985                 990

AAC TTG TTA TAT CGA AGT TTC TAT AGA AGA ACT CCT GAC TTC CTC ACA      3026
Asn Leu Leu Tyr Arg Ser Phe Tyr Arg Arg Thr Pro Asp Phe Leu Thr
                995                 1000                1005

GAG GCT ATA GTT CAC TCT GTG TTC ATA CTT AGT TAT TAT ACA AAC CAT      3074
Glu Ala Ile Val His Ser Val Phe Ile Leu Ser Tyr Tyr Thr Asn His
            1010                1015                1020

GAC TTA AAA GAT AAA CTT CAA GAT CTG TCA GAT GAT AGA TTG AAT AAG      3122
Asp Leu Lys Asp Lys Leu Gln Asp Leu Ser Asp Asp Arg Leu Asn Lys
        1025                1030                1035

TTC TTA ACA TGC ATA ATC ACG TTT GAC AAA GAC CCT AAT GCT GAA TTC      3170
Phe Leu Thr Cys Ile Ile Thr Phe Asp Lys Asp Pro Asn Ala Glu Phe
    1040                1045                1050

GTA ACA TTG ATG AGA GAT CCT CAA GCT TTA GGG TCT GAG AGA CAA GCT      3218
Val Thr Leu Met Arg Asp Pro Gln Ala Leu Gly Ser Glu Arg Gln Ala
1055                1060                1065                1070

AAA ATT ACT AGC GAA ATC AAT AGA CTG GCA GTT ACA GAG GTT TTG AGT      3266
Lys Ile Thr Ser Glu Ile Asn Arg Leu Ala Val Thr Glu Val Leu Ser
                1075                1080                1085

ACA GCT CCA AAC AAA ATA TTC TCC AAA AGT GCA CAA CAT TAT ACT ACT      3314
Thr Ala Pro Asn Lys Ile Phe Ser Lys Ser Ala Gln His Tyr Thr Thr
            1090                1095                1100

ACA GAG ATA GAT CTA AAT GAT ATT ATG CAA AAT ATA GAA CCT ACA TAT      3362
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Thr Glu Ile Asp Leu Asn Asp Ile Met Gln Asn Ile Glu Pro Thr Tyr
        1105                1110                1115

CCT CAT GGG CTA AGA GTT GTT TAT GAA AGT TTA CCC TTT TAT AAA GCA      3410
Pro His Gly Leu Arg Val Val Tyr Glu Ser Leu Pro Phe Tyr Lys Ala
    1120                1125                1130

GAG AAA ATA GTA AAT CTT ATA TCA GGT ACA AAA TCT ATA ACT AAC ATA      3458
Glu Lys Ile Val Asn Leu Ile Ser Gly Thr Lys Ser Ile Thr Asn Ile
1135                1140                1145                1150

CTG GAA AAA ACT TCT GCC ATA GAC TTA ACA GAT ATT GAT AGA GCC ACT      3506
Leu Glu Lys Thr Ser Ala Ile Asp Leu Thr Asp Ile Asp Arg Ala Thr
                1155                1160                1165

GAG ATG ATG AGG AAA AAC ATA ACT TTG CTT ATA AGG ATA CTT CCA TTG      3554
Glu Met Met Arg Lys Asn Ile Thr Leu Leu Ile Arg Ile Leu Pro Leu
            1170                1175                1180

GAT TGT AAC AGA GAT AAA AGA GAG ATA TTG AGT ATG GAA AAC CTA AGT      3602
Asp Cys Asn Arg Asp Lys Arg Glu Ile Leu Ser Met Glu Asn Leu Ser
        1185                1190                1195

ATT ACT GAA TTA AGC AAA TAT GTT AGG GAA AGA TCT TGG TCT TTA TCC      3650
Ile Thr Glu Leu Ser Lys Tyr Val Arg Glu Arg Ser Trp Ser Leu Ser
    1200                1205                1210

AAT ATA GTT GGT GTT ACA TCA CCC AGT ATC ATG TAT ACA ATG GAC ATC      3698
Asn Ile Val Gly Val Thr Ser Pro Ser Ile Met Tyr Thr Met Asp Ile
1215                1220                1225                1230

AAA TAT ACT ACA AGC ACT ATA TCT AGT GGC ATA ATT ATA GAG AAA TAT      3746
Lys Tyr Thr Thr Ser Thr Ile Ser Ser Gly Ile Ile Ile Glu Lys Tyr
                1235                1240                1245

AAT GTT AAC AGT TTA ACA CGT GGT GAG AGA GGA CCC ACT AAA CCA TGG      3794
Asn Val Asn Ser Leu Thr Arg Gly Glu Arg Gly Pro Thr Lys Pro Trp
            1250                1255                1260

GTT GGT TCA TCT ACA CAA GAG AAA AAA ACA ATG CCA GTT TAT AAT AGA      3842
Val Gly Ser Ser Thr Gln Glu Lys Lys Thr Met Pro Val Tyr Asn Arg
        1265                1270                1275

CAA GTC TTA ACC AAA AAA CAG AGA GAT CAA ATA GAT CTA TTA GCA AAA      3890
Gln Val Leu Thr Lys Lys Gln Arg Asp Gln Ile Asp Leu Leu Ala Lys
    1280                1285                1290

TTG GAT TGG GTG TAT GCA TCT ATA GAT AAC AAG GAT GAA TTC ATG GAA      3938
Leu Asp Trp Val Tyr Ala Ser Ile Asp Asn Lys Asp Glu Phe Met Glu
1295                1300                1305                1310

GAA CTC AGC ATA GGA ACC CTT GGG TTA ACA TAT GAA AAG GCC AAG AAA      3986
Glu Leu Ser Ile Gly Thr Leu Gly Leu Thr Tyr Glu Lys Ala Lys Lys
                1315                1320                1325
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TTA TTT CCA CAA TAT TTA AGT GTC AAT TAT TTG CAT CGC CTT ACA GTC      4034
Leu Phe Pro Gln Tyr Leu Ser Val Asn Tyr Leu His Arg Leu Thr Val
            1330                1335                1340

AGT AGT AGA CCA TGT GAA TTC CCT GCA TCA ATA CCA GCT TAT AGA ACA      4082
Ser Ser Arg Pro Cys Glu Phe Pro Ala Ser Ile Pro Ala Tyr Arg Thr
        1345                1350                1355

ACA AAT TAT CAC TTT GAC ACT AGC CCT ATT AAT CGC ATA TTA ACA GAA      4130
Thr Asn Tyr His Phe Asp Thr Ser Pro Ile Asn Arg Ile Leu Thr Glu
    1360                1365                1370

AAG TAT GGT GAT GAA GAT ATT GAC ATA GTA TTC CAA AAC TGT ATA AGC      4178
Lys Tyr Gly Asp Glu Asp Ile Asp Ile Val Phe Gln Asn Cys Ile Ser
1375                1380                1385                1390

TTT GGC CTT AGT TTA ATG TCA GTA GTA GAA CAA TTT ACT AAT GTA TGT      4226
Phe Gly Leu Ser Leu Met Ser Val Val Glu Gln Phe Thr Asn Val Cys
                1395                1400                1405

CCT AAC AGA ATT ATT CTC ATA CCT AAG CTT AAT GAG ATA CAT TTG ATG      4274
Pro Asn Arg Ile Ile Leu Ile Pro Lys Leu Asn Glu Ile His Leu Met
            1410                1415                1420

AAA CCT CCC ATA TTC ACA GGT GAT GTT GAT ATT CAC AAG TTA AAA CAA      4322
Lys Pro Pro Ile Phe Thr Gly Asp Val Asp Ile His Lys Leu Lys Gln
        1425                1430                1435

GTG ATA CAA AAA CAG CAT ATG TTT TTA CCA GAC AAA ATA AGT TTG ACT      4370
Val Ile Gln Lys Gln His Met Phe Leu Pro Asp Lys Ile Ser Leu Thr
    1440                1445                1450

CAA TAT GTG GAA TTA TTC TTA AGT AAT AAA ACA CTC AAA TCT GGA TCT      4418
Gln Tyr Val Glu Leu Phe Leu Ser Asn Lys Thr Leu Lys Ser Gly Ser
1455                1460                1465                1470

CAT GTT AAT TCT AAT TTA ATA TTG GCA CAT AAA ATA TCT GAC TAT TTT      4466
His Val Asn Ser Asn Leu Ile Leu Ala His Lys Ile Ser Asp Tyr Phe
                1475                1480                1485

CAT AAT ACT TAC ATT TTA AGT ACT AAT TTA GCT GGA CAT TGG ATT CTG      4514
His Asn Thr Tyr Ile Leu Ser Thr Asn Leu Ala Gly His Trp Ile Leu
            1490                1495                1500

ATT ATA CAA CTT ATG AAA GAT TCT AAA GGT ATT TTT GAA AAA GAT TGG      4562
Ile Ile Gln Leu Met Lys Asp Ser Lys Gly Ile Phe Glu Lys Asp Trp
        1505                1510                1515

GGA GAG GGA TAT ATA ACT GAT CAT ATG TTT ATT AAT TTG AAA GTT TTC      4610
Gly Glu Gly Tyr Ile Thr Asp His Met Phe Ile Asn Leu Lys Val Phe
    1520                1525                1530

TTC AAT GCT TAT AAG ACC TAT CTC TTG TGT TTT CAT AAA GGT TAT GGC      4658
Phe Asn Ala Tyr Lys Thr Tyr Leu Leu Cys Phe His Lys Gly Tyr Gly
1535                1540                1545                1550

AAA GCA AAG CTG GAG TGT GAT ATG AAC ACT TCA GAT CTT CTA TGT GTA      4706
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Lys Ala Lys Leu Glu Cys Asp Met Asn Thr Ser Asp Leu Leu Cys Val
                1555                1560                1565

TTG GAA TTA ATA GAC AGT AGT TAT TGG AAG TCT ATG TCT AAG GTA TTT      4754
Leu Glu Leu Ile Asp Ser Ser Tyr Trp Lys Ser Met Ser Lys Val Phe
            1570                1575                1580

TTA GAA CAA AAA GTT ATC AAA TAC ATT CTT AGC CAA GAT GCA AGT TTA      4802
Leu Glu Gln Lys Val Ile Lys Tyr Ile Leu Ser Gln Asp Ala Ser Leu
        1585                1590                1595

CAT AGA GTA AAA GGA TGT CAT AGC TTC AAA TTA TGG TTT CTT AAA CGT      4850
His Arg Val Lys Gly Cys His Ser Phe Lys Leu Trp Phe Leu Lys Arg
    1600                1605                1610

CTT AAT GTA GCA GAA TTC ACA GTT TGC CCT TGG GTT GTT AAC ATA GAT      4898
Leu Asn Val Ala Glu Phe Thr Val Cys Pro Trp Val Val Asn Ile Asp
1615                1620                1625                1630

TAT CAT CCA ACA CAT ATG AAA GCA ATA TTA ACT TAT ATA GAT CTT GTT      4946
Tyr His Pro Thr His Met Lys Ala Ile Leu Thr Tyr Ile Asp Leu Val
                1635                1640                1645

AGA ATG GGA TTG ATA AAT ATA GAT AGA ATA CAC ATT AAA AAT AAA CAC      4994
Arg Met Gly Leu Ile Asn Ile Asp Arg Ile His Ile Lys Asn Lys His
            1650                1655                1660

AAA TTC AAT GAT GAA TTT TAT ACT TCT AAT CTC TTC TAC ATT AAT TAT      5042
Lys Phe Asn Asp Glu Phe Tyr Thr Ser Asn Leu Phe Tyr Ile Asn Tyr
        1665                1670                1675

AAC TTC TCA GAT AAT ACT CAT CTA TTA ACT AAA CAT ATA AGG ATT GCT      5090
Asn Phe Ser Asp Asn Thr His Leu Leu Thr Lys His Ile Arg Ile Ala
    1680                1685                1690

AAT TCT GAA TTA GAA AAT AAT TAC AAC AAA TTA TAT CAT CCT ACA CCA      5138
Asn Ser Glu Leu Glu Asn Asn Tyr Asn Lys Leu Tyr His Pro Thr Pro
1695                1700                1705                1710

GAA ACC CTA GAG AAT ATA CTA GCC AAT CCG ATT AAA AGT AAT GAC AAA      5186
Glu Thr Leu Glu Asn Ile Leu Ala Asn Pro Ile Lys Ser Asn Asp Lys
                1715                1720                1725

AAG ACA CTG AAT GAC TAT TGT ATA GGT AAA AAT GTT GAC TCA ATA ATG      5234
Lys Thr Leu Asn Asp Tyr Cys Ile Gly Lys Asn Val Asp Ser Ile Met
            1730                1735                1740

TTA CCA TTG TTA TCT AAT AAG AAG CTT ATT AAA TCG TCT GCA ATG ATT      5282
Leu Pro Leu Leu Ser Asn Lys Lys Leu Ile Lys Ser Ser Ala Met Ile
        1745                1750                1755

AGA ACC AAT TAC AGC AAA CAA GAT TTG TAT AAT TTA TTC CCT ATG GTT      5330
Arg Thr Asn Tyr Ser Lys Gln Asp Leu Tyr Asn Leu Phe Pro Met Val
    1760                1765                1770
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GTG ATT GAT AGA ATT ATA GAT CAT TCA GGC AAT ACA GCC AAA TCC AAC      5378
Val Ile Asp Arg Ile Ile Asp His Ser Gly Asn Thr Ala Lys Ser Asn
1775                 1780                 1785                 1790

CAA CTT TAC ACT ACT ACT TCC CAC CAA ATA TCT TTA GTG CAC AAT AGC      5426
Gln Leu Tyr Thr Thr Thr Ser His Gln Ile Ser Leu Val His Asn Ser
                1795                 1800                 1805

ACA TCA CTT TAC TGC ATG CTT CCT TGG CAT CAT ATT AAT AGA TTC AAT      5474
Thr Ser Leu Tyr Cys Met Leu Pro Trp His His Ile Asn Arg Phe Asn
            1810                 1815                 1820

TTT GTA TTT AGT TCT ACA GGT TGT AAA ATT AGT ATA GAG TAT ATT TTA      5522
Phe Val Phe Ser Ser Thr Gly Cys Lys Ile Ser Ile Glu Tyr Ile Leu
        1825                 1830                 1835

AAA GAT CTT AAA ATT AAA GAT CCC AAT TGT ATA GCA TTC ATA GGT GAA      5570
Lys Asp Leu Lys Ile Lys Asp Pro Asn Cys Ile Ala Phe Ile Gly Glu
    1840                 1845                 1850

GGA GCA GGG AAT TTA TTA TTG CGT ACA GTA GTG GAA CTT CAT CCT GAC      5618
Gly Ala Gly Asn Leu Leu Leu Arg Thr Val Val Glu Leu His Pro Asp
1855                 1860                 1865                 1870

ATA AGA TAT ATT TAC AGA AGT CTG AAA GAT TGC AAT GAT CAT AGT TTA      5666
Ile Arg Tyr Ile Tyr Arg Ser Leu Lys Asp Cys Asn Asp His Ser Leu
                1875                 1880                 1885

CCT ATT GAG TTT TTA AGG CTG TAC AAT GGA CAT ATC AAC ATT GAT TAT      5714
Pro Ile Glu Phe Leu Arg Leu Tyr Asn Gly His Ile Asn Ile Asp Tyr
            1890                 1895                 1900

GGT GAA AAT TTG ACC ATT CCT GCT ACA GAT GCA ACC AAC AAC ATT CAT      5762
Gly Glu Asn Leu Thr Ile Pro Ala Thr Asp Ala Thr Asn Asn Ile His
        1905                 1910                 1915

TGG TCT TAT TTA CAT ATA AAG TTT GCT GAA CCT ATC AGT CTT TTT GTC      5810
Trp Ser Tyr Leu His Ile Lys Phe Ala Glu Pro Ile Ser Leu Phe Val
    1920                 1925                 1930

TGT GAT GCC GAA TTG TCT GTA ACA GTC AAC TGG AGT AAA ATT ATA ATA      5858
Cys Asp Ala Glu Leu Ser Val Thr Val Asn Trp Ser Lys Ile Ile Ile
1935                 1940                 1945                 1950

GAA TGG AGC AAG CAT GTA AGA AAG TGC AAG TAC TGT TCC TCA GTT AAT      5906
Glu Trp Ser Lys His Val Arg Lys Cys Lys Tyr Cys Ser Ser Val Asn
                1955                 1960                 1965

AAA TGT ATG TTA ATA GTA AAA TAT CAT GCT CAA GAT GAT ATT GAT TTC      5954
Lys Cys Met Leu Ile Val Lys Tyr His Ala Gln Asp Asp Ile Asp Phe
            1970                 1975                 1980

AAA TTA GAC AAT ATA ACT ATA TTA AAA ACT TAT GTA TGC TTA GGC AGT      6002
Lys Leu Asp Asn Ile Thr Ile Leu Lys Thr Tyr Val Cys Leu Gly Ser
        1985                 1990                 1995

AAG TTA AAG GGA TCG GAG GTT TAC TTA GTC CTT ACA ATA GGT CCT GCG      6050
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Lys Leu Lys Gly Ser Glu Val Tyr Leu Val Leu Thr Ile Gly Pro Ala
    2000                2005                2010

AAT ATA TTC CCA GTA TTT AAT GTA GTA CAA AAT GCT AAA TTG ATA CTA      6098
Asn Ile Phe Pro Val Phe Asn Val Val Gln Asn Ala Lys Leu Ile Leu
2015                2020                2025                2030

TCA AGA ACC AAA AAT TTC ATC ATG CCT AAG AAA GCT GAT AAA GAG TCT      6146
Ser Arg Thr Lys Asn Phe Ile Met Pro Lys Lys Ala Asp Lys Glu Ser
                2035                2040                2045

ATT GAT GCA AAT ATT AAA AGT TTG ATA CCC TTT CTT TGT TAC CCT ATA      6194
Ile Asp Ala Asn Ile Lys Ser Leu Ile Pro Phe Leu Cys Tyr Pro Ile
            2050                2055                2060

ACA AAA AAA GGA ATT AAT ACT GCA TTG TCA AAA CTA AAG AGT GTT GTT      6242
Thr Lys Lys Gly Ile Asn Thr Ala Leu Ser Lys Leu Lys Ser Val Val
        2065                2070                2075

AGT GGA GAT ATA CTA TCA TAT TCT ATA GCT GGA CGT AAT GAA GTT TTC      6290
Ser Gly Asp Ile Leu Ser Tyr Ser Ile Ala Gly Arg Asn Glu Val Phe
    2080                2085                2090

AGC AAT AAA CTT ATA AAT CAT AAG CAT ATG AAC ATC TTA AAA TGG TTC      6338
Ser Asn Lys Leu Ile Asn His Lys His Met Asn Ile Leu Lys Trp Phe
2095                2100                2105                2110

AAT CAT GTT TTA AAT TTC AGA TCA ACA GAA CTA AAC TAT AAC CAT TTA      6386
Asn His Val Leu Asn Phe Arg Ser Thr Glu Leu Asn Tyr Asn His Leu
                2115                2120                2125

TAT ATG GTA GAA TCT ACA TAT CCT TAC CTA AGT GAA TTG TTA AAC AGC      6434
Tyr Met Val Glu Ser Thr Tyr Pro Tyr Leu Ser Glu Leu Leu Asn Ser
            2130                2135                2140

TTG ACA ACC AAT GAA CTT AAA AAA CTG ATT AAA ATC ACA GGT AGT CTG      6482
Leu Thr Thr Asn Glu Leu Lys Lys Leu Ile Lys Ile Thr Gly Ser Leu
        2145                2150                2155

TTA TAC AAC TTT CAT AAT GAA T AATGAATAAA GATCTTATAA TAAAAATTCC       6534
Leu Tyr Asn Phe His Asn Glu
    2160                216

CATAGCTATA CACTAACACT GTATTCAATT ATAGTTATTA AAAA                     6578
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2165 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Asp Pro Ile Ile Asn Gly Asn Ser Ala Asn Val Tyr Leu Thr Asp
1 5 10 15

Gly Tyr Leu Lys Gly Val Ile Ser Phe Ser Glu Cys Asn Ala Leu Gly
20 25 30

Ser Tyr Ile Phe Asn Gly Pro Tyr Leu Lys Asn Asp Tyr Thr Asn Leu
35 40 45

Ile Ser Arg Gln Asn Pro Leu Ile Glu His Met Asn Leu Lys Lys Leu
50 55 60

Asn Ile Thr Gln Ser Leu Ile Ser Lys Tyr His Lys Gly Glu Ile Lys
65 70 75 80

Leu Glu Glu Pro Thr Tyr Phe Gln Ser Leu Leu Met Thr Tyr Lys Ser
85 90 95

Met Thr Ser Ser Glu Gln Ile Ala Thr Thr Asn Leu Leu Lys Lys Ile
100 105 110

Ile Arg Arg Ala Ile Glu Ile Ser Asp Val Lys Val Tyr Ala Ile Leu
115 120 125

Asn Lys Leu Gly Leu Lys Glu Lys Asp Lys Ile Lys Ser Asn Asn Gly
130 135 140

Gln Asp Glu Asp Asn Ser Val Ile Thr Thr Ile Ile Lys Asp Asp Ile
145 150 155 160

Leu Ser Ala Val Lys Asp Asn Gln Ser His Leu Lys Ala Asp Lys Asn
165 170 175

His Ser Thr Lys Gln Lys Asp Thr Ile Lys Thr Thr Leu Leu Lys Lys
180 185 190

Leu Met Cys Ser Met Gln His Pro Pro Ser Trp Leu Ile His Trp Phe
195 200 205

Asn Leu Tyr Thr Lys Leu Asn Asn Ile Leu Thr Gln Tyr Arg Ser Asn
210 215 220

Glu Val Lys Asn His Gly Phe Thr Leu Ile Asp Asn Gln Thr Leu Ser
225 230 235 240

Gly Phe Gln Phe Ile Leu Asn Gln Tyr Gly Cys Ile Val Tyr His Lys
245 250 255

Glu Leu Lys Arg Ile Thr Val Thr Thr Tyr Asn Gln Phe Leu Thr Trp
260 265 270

Lys Asp Ile Ser Leu Ser Arg Leu Asn Val Cys Leu Ile Thr Trp Ile
275 280 285

Ser Asn Cys Leu Asn Thr Leu Asn Lys Ser Leu Gly Leu Arg Cys Gly
290 295 300

Phe Asn Asn Val Ile Leu Thr Gln Leu Phe Leu Tyr Gly Asp Cys Ile
305 310 315 320

Leu Lys Leu Phe His Asn Glu Gly Phe Tyr Ile Ile Lys Glu Val Glu
325 330 335

Gly Phe Ile Met Ser Leu Ile Leu Asn Ile Thr Glu Glu Asp Gln Phe
340 345 350

Arg Lys Arg Phe Tyr Asn Ser Met Leu Asn Asn Ile Thr Asp Ala Ala
355 360 365

Asn Lys Ala Gln Lys Asn Leu Leu Ser Arg Val Cys His Thr Leu Leu
370 375 380

Asp Lys Thr Val Ser Asp Asn Ile Ile Asn Gly Arg Trp Ile Ile Leu
385 390 395 400

Leu Ser Lys Phe Leu Lys Leu Ile Lys Leu Ala Gly Asp Asn Asn Leu
405 410 415

Asn Asn Leu Ser Glu Leu Tyr Phe Leu Phe Arg Ile Phe Gly His Pro
420 425 430

Met Val Asp Glu Arg Gln Ala Met Asp Ala Val Lys Ile Asn Cys Asn
435 440 445

Glu Thr Lys Phe Tyr Leu Leu Ser Ser Leu Ser Met Leu Arg Gly Ala
450 455 460

Phe Ile Tyr Arg Ile Ile Lys Gly Phe Val Asn Asn Tyr Asn Arg Trp
465 470 475 480

Pro Thr Leu Arg Asn Ala Ile Val Leu Pro Leu Arg Trp Leu Thr Tyr
485 490 495

Tyr Lys Leu Asn Thr Tyr Pro Ser Leu Leu Glu Leu Thr Glu Arg Asp
500 505 510

Leu Ile Val Leu Ser Gly Leu Arg Phe Tyr Arg Glu Phe Arg Leu Pro
515 520 525

Lys Lys Val Asp Leu Glu Met Ile Ile Asn Asp Lys Ala Ile Ser Pro
530 535 540

Pro Lys Asn Leu Ile Trp Thr Ser Phe Pro Arg Asn Tyr Met Pro Ser
545 550 555 560

His Ile Gln Asn Tyr Ile Glu His Glu Lys Leu Lys Phe Ser Glu Ser
565 570 575

Asp Lys Ser Arg Arg Val Leu Glu Tyr Tyr Leu Arg Asp Asn Lys Phe
580 585 590

Asn Glu Cys Asp Leu Tyr Asn Cys Val Val Asn Gln Ser Tyr Leu Asn
595 600 605

Asn Pro Asn His Val Val Ser Leu Thr Gly Lys Glu Arg Glu Leu Ser
610 615 620

Val Gly Arg Met Phe Ala Met Gln Pro Gly Met Phe Arg Gln Val Gln
625 630 635 640

Ile Leu Ala Glu Lys Met Ile Ala Glu Asn Ile Leu Gln Phe Phe Pro
645 650 655

Glu Ser Leu Thr Arg Tyr Gly Asp Leu Glu Leu Gln Lys Ile Leu Glu
660 665 670

Leu Lys Ala Gly Ile Ser Asn Lys Ser Asn Arg Tyr Asn Asp Asn Tyr
675 680 685

Asn Asn Tyr Ile Ser Lys Cys Ser Ile Ile Thr Asp Leu Ser Lys Phe
690 695 700

Asn Gln Ala Phe Arg Tyr Glu Thr Ser Cys Ile Cys Ser Asp Val Leu
705 710 715 720

Asp Glu Leu His Gly Val Gln Ser Leu Phe Ser Trp Leu His Leu Thr
725 730 735

Ile Pro His Val Thr Ile Ile Cys Thr Tyr Arg His Ala Pro Pro Tyr
740 745 750

Ile Gly Asp His Ile Val Asp Leu Asn Asn Val Asp Glu Gln Ser Gly
755 760 765

Leu Tyr Arg Tyr His Met Gly Gly Ile Glu Gly Trp Cys Gln Lys Leu
770 775 780

Trp Thr Ile Glu Ala Ile Ser Leu Leu Asp Leu Ile Ser Leu Lys Gly
785 790 795 800

Lys Phe Ser Ile Thr Ala Leu Ile Asn Gly Asp Asn Gln Ser Ile Asp
805 810 815

Ile Ser Lys Pro Ile Arg Leu Met Glu Gly Gln Thr His Ala Gln Ala
820 825 830

Asp Tyr Leu Leu Ala Leu Asn Ser Leu Lys Leu Leu Tyr Lys Glu Tyr
835 840 845

Ala Gly Ile Gly His Lys Leu Lys Gly Thr Glu Thr Tyr Ile Ser Arg
850 855 860

Asp Met Gln Phe Met Ser Lys Thr Ile Gln His Asn Gly Val Tyr Tyr
865 870 875 880

Pro Ala Ser Ile Lys Lys Val Leu Arg Val Gly Pro Trp Ile Asn Thr
885 890 895

Ile Leu Asp Asp Phe Lys Val Ser Leu Glu Ser Ile Gly Ser Leu Thr
900 905 910

Gln Glu Leu Glu Tyr Arg Gly Glu Ser Leu Leu Cys Ser Leu Ile Phe
915 920 925

Arg Asn Val Trp Leu Tyr Asn Gln Ile Ala Leu Gln Leu Lys Asn His
930 935 940

Ala Leu Cys Asn Asn Lys Leu Tyr Leu Asp Ile Leu Lys Val Leu Lys
945 950 955 960

His Leu Lys Thr Phe Phe Asn Leu Asp Asn Ile Asp Thr Ala Leu Thr
965 970 975

Leu Tyr Met Asn Leu Pro Met Leu Phe Gly Gly Gly Asp Pro Asn Leu
980 985 990

Leu Tyr Arg Ser Phe Tyr Arg Arg Thr Pro Asp Phe Leu Thr Glu Ala
995 1000 1005

Ile Val His Ser Val Phe Ile Leu Ser Tyr Tyr Thr Asn His Asp Leu
1010 1015 1020

Lys Asp Lys Leu Gln Asp Leu Ser Asp Asp Arg Leu Asn Lys Phe Leu
1025 1030 1035 1040

Thr Cys Ile Ile Thr Phe Asp Lys Asp Pro Asn Ala Glu Phe Val Thr
1045 1050 1055

Leu Met Arg Asp Pro Gln Ala Leu Gly Ser Glu Arg Gln Ala Lys Ile
1060 1065 1070

Thr Ser Glu Ile Asn Arg Leu Ala Val Thr Glu Val Leu Ser Thr Ala
1075 1080 1085

Pro Asn Lys Ile Phe Ser Lys Ser Ala Gln His Tyr Thr Thr Thr Glu
1090 1095 1100

Ile Asp Leu Asn Asp Ile Met Gln Asn Ile Glu Pro Thr Tyr Pro His
1105 1110 1115 1120

Gly Leu Arg Val Val Tyr Glu Ser Leu Pro Phe Tyr Lys Ala Glu Lys
1125 1130 1135

Ile Val Asn Leu Ile Ser Gly Thr Lys Ser Ile Thr Asn Ile Leu Glu
1140 1145 1150

Lys Thr Ser Ala Ile Asp Leu Thr Asp Ile Asp Arg Ala Thr Glu Met
1155 1160 1165

Met Arg Lys Asn Ile Thr Leu Leu Ile Arg Ile Leu Pro Leu Asp Cys
1170 1175 1180

Asn Arg Asp Lys Arg Glu Ile Leu Ser Met Glu Asn Leu Ser Ile Thr
1185 1190 1195 1200

Glu Leu Ser Lys Tyr Val Arg Glu Arg Ser Trp Ser Leu Ser Asn Ile
1205 1210 1215

Val Gly Val Thr Ser Pro Ser Ile Met Tyr Thr Met Asp Ile Lys Tyr
1220 1225 1230

Thr Thr Ser Thr Ile Ser Ser Gly Ile Ile Ile Glu Lys Tyr Asn Val
1235 1240 1245

Asn Ser Leu Thr Arg Gly Glu Arg Gly Pro Thr Lys Pro Trp Val Gly
1250 1255 1260

Ser Ser Thr Gln Glu Lys Lys Thr Met Pro Val Tyr Asn Arg Gln Val
1265 1270 1275 1280

Leu Thr Lys Lys Gln Arg Asp Gln Ile Asp Leu Leu Ala Lys Leu Asp
1285 1290 1295

Trp Val Tyr Ala Ser Ile Asp Asn Lys Asp Glu Phe Met Glu Glu Leu
1300 1305 1310

Ser Ile Gly Thr Leu Gly Leu Thr Tyr Glu Lys Ala Lys Lys Leu Phe
1315 1320 1325

Pro Gln Tyr Leu Ser Val Asn Tyr Leu His Arg Leu Thr Val Ser Ser
1330 1335 1340

Arg Pro Cys Glu Phe Pro Ala Ser Ile Pro Ala Tyr Arg Thr Thr Asn
1345 1350 1355 1360

Tyr His Phe Asp Thr Ser Pro Ile Asn Arg Ile Leu Thr Glu Lys Tyr
1365 1370 1375

Gly Asp Glu Asp Ile Asp Ile Val Phe Gln Asn Cys Ile Ser Phe Gly
1380 1385 1390

Leu Ser Leu Met Ser Val Val Glu Gln Phe Thr Asn Val Cys Pro Asn
1395 1400 1405

Arg Ile Ile Leu Ile Pro Lys Leu Asn Glu Ile His Leu Met Lys Pro
1410 1415 1420

Pro Ile Phe Thr Gly Asp Val Asp Ile His Lys Leu Lys Gln Val Ile
1425 1430 1435 1440

Gln Lys Gln His Met Phe Leu Pro Asp Lys Ile Ser Leu Thr Gln Tyr
1445 1450 1455

Val Glu Leu Phe Leu Ser Asn Lys Thr Leu Lys Ser Gly Ser His Val
1460 1465 1470

Asn Ser Asn Leu Ile Leu Ala His Lys Ile Ser Asp Tyr Phe His Asn
1475 1480 1485

Thr Tyr Ile Leu Ser Thr Asn Leu Ala Gly His Trp Ile Leu Ile Ile
1490 1495 1500

Gln Leu Met Lys Asp Ser Lys Gly Ile Phe Glu Lys Asp Trp Gly Glu
1505 1510 1515 1520

Gly Tyr Ile Thr Asp His Met Phe Ile Asn Leu Lys Val Phe Phe Asn
1525 1530 1535

Ala Tyr Lys Thr Tyr Leu Leu Cys Phe His Lys Gly Tyr Gly Lys Ala
1540 1545 1550

Lys Leu Glu Cys Asp Met Asn Thr Ser Asp Leu Leu Cys Val Leu Glu
1555 1560 1565

Leu Ile Asp Ser Ser Tyr Trp Lys Ser Met Ser Lys Val Phe Leu Glu
1570 1575 1580

Gln Lys Val Ile Lys Tyr Ile Leu Ser Gln Asp Ala Ser Leu His Arg
1585 1590 1595 1600

Val Lys Gly Cys His Ser Phe Lys Leu Trp Phe Leu Lys Arg Leu Asn
1605 1610 1615

Val Ala Glu Phe Thr Val Cys Pro Trp Val Val Asn Ile Asp Tyr His
1620 1625 1630

Pro Thr His Met Lys Ala Ile Leu Thr Tyr Ile Asp Leu Val Arg Met
1635 1640 1645

Gly Leu Ile Asn Ile Asp Arg Ile His Ile Lys Asn Lys His Lys Phe
1650 1655 1660

Asn Asp Glu Phe Tyr Thr Ser Asn Leu Phe Tyr Ile Asn Tyr Asn Phe
1665 1670 1675 1680

Ser Asp Asn Thr His Leu Leu Thr Lys His Ile Arg Ile Ala Asn Ser
1685 1690 1695

Glu Leu Glu Asn Asn Tyr Asn Lys Leu Tyr His Pro Thr Pro Glu Thr
1700 1705 1710

Leu Glu Asn Ile Leu Ala Asn Pro Ile Lys Ser Asn Asp Lys Lys Thr
1715 1720 1725

Leu Asn Asp Tyr Cys Ile Gly Lys Asn Val Asp Ser Ile Met Leu Pro
1730 1735 1740

Leu Leu Ser Asn Lys Lys Leu Ile Lys Ser Ser Ala Met Ile Arg Thr
1745 1750 1755 1760

Asn Tyr Ser Lys Gln Asp Leu Tyr Asn Leu Phe Pro Met Val Val Ile
1765 1770 1775

Asp Arg Ile Ile Asp His Ser Gly Asn Thr Ala Lys Ser Asn Gln Leu
1780 1785 1790

Tyr Thr Thr Thr Ser His Gln Ile Ser Leu Val His Asn Ser Thr Ser
1795 1800 1805

Leu Tyr Cys Met Leu Pro Trp His His Ile Asn Arg Phe Asn Phe Val
1810 1815 1820

Phe Ser Ser Thr Gly Cys Lys Ile Ser Ile Glu Tyr Ile Leu Lys Asp
1825 1830 1835 1840

Leu Lys Ile Lys Asp Pro Asn Cys Ile Ala Phe Ile Gly Glu Gly Ala
1845 1850 1855

Gly Asn Leu Leu Leu Arg Thr Val Val Glu Leu His Pro Asp Ile Arg
1860 1865 1870

Tyr Ile Tyr Arg Ser Leu Lys Asp Cys Asn Asp His Ser Leu Pro Ile
1875 1880 1885

Glu Phe Leu Arg Leu Tyr Asn Gly His Ile Asn Ile Asp Tyr Gly Glu
1890 1895 1900

Asn Leu Thr Ile Pro Ala Thr Asp Ala Thr Asn Asn Ile His Trp Ser
1905 1910 1915 1920

Tyr Leu His Ile Lys Phe Ala Glu Pro Ile Ser Leu Phe Val Cys Asp
1925 1930 1935

Ala Glu Leu Ser Val Thr Val Asn Trp Ser Lys Ile Ile Ile Glu Trp
1940 1945 1950

Ser Lys His Val Arg Lys Cys Lys Tyr Cys Ser Ser Val Asn Lys Cys
1955 1960 1965

Met Leu Ile Val Lys Tyr His Ala Gln Asp Asp Ile Asp Phe Lys Leu
1970 1975 1980

Asp Asn Ile Thr Ile Leu Lys Thr Tyr Val Cys Leu Gly Ser Lys Leu
1985 1990 1995 2000

Lys Gly Ser Glu Val Tyr Leu Val Leu Thr Ile Gly Pro Ala Asn Ile
2005 2010 2015

Phe Pro Val Phe Asn Val Val Gln Asn Ala Lys Leu Ile Leu Ser Arg
2020 2025 2030

Thr Lys Asn Phe Ile Met Pro Lys Lys Ala Asp Lys Glu Ser Ile Asp
2035 2040 2045

Ala Asn Ile Lys Ser Leu Ile Pro Phe Leu Cys Tyr Pro Ile Thr Lys
2050 2055 2060

Lys Gly Ile Asn Thr Ala Leu Ser Lys Leu Lys Ser Val Val Ser Gly
2065 2070 2075 2080

Asp Ile Leu Ser Tyr Ser Ile Ala Gly Arg Asn Glu Val Phe Ser Asn
2085 2090 2095

Lys Leu Ile Asn His Lys His Met Asn Ile Leu Lys Trp Phe Asn His
2100 2105 2110

Val Leu Asn Phe Arg Ser Thr Glu Leu Asn Tyr Asn His Leu Tyr Met
2115 2120 2125

Val Glu Ser Thr Tyr Pro Tyr Leu Ser Glu Leu Leu Asn Ser Leu Thr
2130 2135 2140

Thr Asn Glu Leu Lys Lys Leu Ile Lys Ile Thr Gly Ser Leu Leu Tyr
2145 2150 2155 2160

Asn Phe His Asn Glu
2165

## Claims

1. A pure, recombinant, replicating and spreading non-segmented RNA virus particle, comprising: i) a non-segmented virus RNA dependent RNA polymerase (L); ii) a non-segmented virus phosphoprotein (P); iii) a non-segmented virus nucleocapsid (N); iv) non-segmented virus structural protein; v) a 3' non-coding RNA sequence; vi) a 3' to 5' RNA coding sequence, which encodes the non-segmented virus L, P, N and non-segmented virus structural proteins required for assembly of budded infectious particles and includes a heterologous gene (X) and vii) a 5' non-coding RNA sequence.

2. A virus particle of claim 1, wherein the non-segmented virus is a paramyxovirus.

3. A virus particle of claim 2, wherein the paramyxovirus is a pneumovirus

4. A virus particle of claim 3, wherein the pneumovirus is a respiratory syncytial virus.

5. A virus particle of claim 4, wherein the respiratory syncytial virus is a human respiratory syncytial virus.

6. A virus particle of claim 4, wherein the respiratory syncytial virus particle is a bovine respiratory syncytial virus.

7. A virus particle of claim 1, wherein the non-segmented virus is a rhabdovirus.

8. A virus particle of claim 7, wherein the rhabdovirus is vesicular stomatitis virus

9. A vaccine comprising the virus particle of claim 1, wherein the heterologous gene (X) encodes at least one pathogen protective epitope.

10. A vaccine of claim 9, wherein the pathogen is selected from the group consisting of a bacteria, mycobacteria, virus, fungi and protozoan.

11. A vaccine of claim 10, wherein the bacteria is selected from the group consisting of intestinal toxin producing *E. coli*, *Hemophilus influenza* type b, *Neisseria*

*meningitidis*, *Salmonella typhi*, *Shigella*, *Streptococcus* Group A, *Streptococcus pneumoniae*, and *Vibrio cholerae*.

12. A vaccine of claim 10, wherein the virus is selected from the group consisting of Dengue virus, Hepatitis A virus, Hepatitis B virus, Japanese encephalitis virus, Parainfluenza virus, Rabies virus, Respiratory Syncytial virus and Rotavirus.

13. A gene therapy vector comprising the virus particle of claim 1, wherein the heterologous gene (X) encodes a protein that supplements a defective or inappropriately expressed protein in a patient.

14. A gene therapy vector of claim 13, wherein the protein is selected from the group consisting of: adenosine deaminase, purine nucleoside phosphorylase, carbonic anyhydrase II, erythropoietin, α or β thalassemia, thrombopoietin, an anti-sickling globin, factor VIII, Factor IX, α-1 antitrypsin, C1 esterase inhibitor, carbanyl phosphate synthetase, ornithine, transcarbamylase, argininosuccinate lyase, arginase, propionyl CoA carboxylase, methylmalonyl CoA mutase, phenylalanine hydroxylase, galactose-1-phosphate uridyl transferase, cystathionine β synthase, branched chain 2-keto acid decarboxylase, galactosidase, glucocerebrosidase, hypoxanthine phosphoribosyltransferase, hexosaminidase, low density lipoprotein receptor, insulin, growth hormones, growth factors, interleukins, interferons, cytokines, colony stimulating factors, cystic fibrosis transmembrane conductance regulator protein, dystrophin, antibodies, antibacterial agents, antiviral agents, antifungal agents, antiprotozoal agents, multidrug resistance, superoxide dismutase and transforming growth factors.

15. A gene therapy vector of claim 14, wherein the virus particle has a respiratory syncytial virus backbone and the protein or oligonucleotide has bioactivity in a subject's lung.

16. A gene therapy vector of claim 15, wherein the protein is selected from the group consisting of: the cystic fibrosis transmembrane conductance regulator (CFTR) protein or a functional fragment thereof, an anti protease (e.g. alpha-1-antitrypsin), a tissue inhibitor of metaloproteinase, an antioxidant (e.g., superoxide dismutase), a cytokine (e.g., an interferon), a mucolytic (e.g., DNase); or a protein which blocks the action of an inflammatory cytokine.

17. A gene therapy vector comprising the virus particle of claim 1, wherein the heterologous gene (X) is an antisense or other biologically active nucleic acid molecule.

18. A pure, recombinant, replicating and non-spreading non-segmented RNA virus particle, comprising: i) a non-segmented virus RNA dependent RNA polymerase (L); ii) a non-segmented virus phosphoprotein (P); iii) a non-segmented virus nucleocapsid (N); iv) non-segmented virus structural protein; v) a 3' non-coding RNA sequence; vi) a 3' to 5' RNA coding sequence, which encodes the non-segmented virus L, P, N but no non-segmented virus structural proteins required for assembly of budded infectious particles and includes a heterologous gene (X) and vii) a 5' non-coding RNA sequence.

19. A virus particle of claim 18, wherein the non-segmented virus is a paramyxovirus.

20. A virus particle of claim 19, wherein the paramyxovirus is a pneumovirus

21. A virus particle of claim 20, wherein the pneumovirus is a respiratory syncytial virus.

22. A virus particle of claim 21, wherein the respiratory syncytial virus is a human respiratory syncytial virus.

23. A virus particle of claim 21, wherein the respiratory syncytial virus particle is a bovine respiratory syncytial virus.

24. A virus particle of claim 18, wherein the non-segmented virus is a rhabdovirus.

25. A virus particle of claim 24, wherein the rhabdovirus is vesicular stomatitis virus

26. A vaccine comprising the virus particle of claim 18, wherein the heterologous gene (X) encodes at least one pathogen protective epitope.

27. A vaccine of claim 26, wherein the pathogen is selected from the group consisting of a bacteria, mycobacteria, virus, fungi and protozoan.

28. A vaccine of claim 27, wherein the bacteria is selected from the group consisting of intestinal toxin producing *E. coli*, *Hemophilus influenza* type b, *Neisseria*

*meningitidis*, *Salmonella typhi*, *Shigella*, *Streptococcus* Group A, *Streptococcus pneumoniae*, and *Vibrio cholerae*.

29. A vaccine of claim 27, wherein the virus is selected from the group consisting of Dengue virus, Hepatitis A virus, Hepatitis B virus, Japanese encephalitis virus, Parainfluenza virus, Rabies virus, Respiratory Syncytial virus and Rotavirus.

30. A gene therapy vector comprising the virus particle of claim 1, wherein the heterologous gene (X) encodes a protein that supplements a defective or inappropriately expressed protein in a patient.

31. A gene therapy vector of claim 30, wherein the protein is selected from the group consisting of: adenosine deaminase, purine nucleoside phosphorylase, carbonic anyhydrase II, erythropoietin, α or β thalassemia, thrombopoietin, an anti-sickling globin, factor VIII, Factor IX, α-1 antitrypsin, C1 esterase inhibitor, carbanyl phosphate synthetase, ornithine, transcarbamylase, argininosuccinate lyase, arginase, propionyl CoA carboxylase, methylmalonyl CoA mutase, phenylalanine hydroxylase, galactose-1-phosphate uridyl transferase, cystathionine β synthase, branched chain 2-keto acid decarboxylase, galactosidase, glucocerebrosidase, hypoxanthine phosphoribosyltransferase, hexosaminidase, low density lipoprotein receptor, insulin, growth hormones, growth factors, interleukins, interferons, cytokines, colony stimulating factors, cystic fibrosis transmembrane conductance regulator protein, dystrophin, antibodies, antibacterial agents, antiviral agents, antifungal agents, antiprotozoal agents, multidrug resistance, superoxide dismutase and transforming growth factors.

32. A gene therapy vector of claim 31, wherein the virus particle has a respiratory syncytial virus backbone and the protein or oligonucleotide has bioactivity in a subject's lung.

33. A gene therapy vector of claim 32, wherein the protein is selected from the group consisting of: the cystic fibrosis transmembrane conductance regulator (CFTR) protein or a functional fragment thereof, an anti protease (e.g. alpha-1-antitrypsin), a tissue inhibitor of metaloproteinase, an antioxidant (e.g., superoxide dismutase), a cytokine (e.g., an interferon), a mucolytic (e.g., DNase); or a protein which blocks the action of an inflammatory cytokine.

34. A gene therapy vector comprising the virus particle of claim 1, wherein the heterologous gene (X) is an antisense or other biologically active nucleic acid molecule.

35. A pure, recombinant, non-segmented RNA virus transcribing particle, comprising: i) a non-segmented virus RNA dependent RNA polymerase (L); ii) a non-segmented virus phosphoprotein (P); iii) a non-segmented virus nucleocapsid (N); iv) non-segmented virus structural protein; v) a 3' non-coding RNA sequence; vi) a 3' to 5' RNA coding sequence, which contains an appropriate transcription initiation sequence and a heterologous gene (X) and vii) a 5' non-coding RNA sequence.

36. A virus particle of claim 35, wherein the non-segmented virus is a paramyxovirus.

37. A virus particle of claim 36, wherein the paramyxovirus is a pneumovirus.

38. A virus particle of claim 37, wherein the pneumovirus is a respiratory syncytial virus.

39. A virus particle of claim 38, wherein the respiratory syncytial virus is a human respiratory syncytial virus.

40. A virus particle of claim 39, wherein the respiratory syncytial virus particle is a bovine respiratory syncytial virus.

41. A virus particle of claim 35, wherein the non-segmented virus is a rhabdovirus.

42. A virus particle of claim 41, wherein the rhabdovirus is vesicular stomatitis virus.

43. A cDNA encoding a functional RSV, RNA dependent, RNA polymerase (L) protein.

44. A cDNA of Claim 43 comprising SEQ. ID. NO: 1.

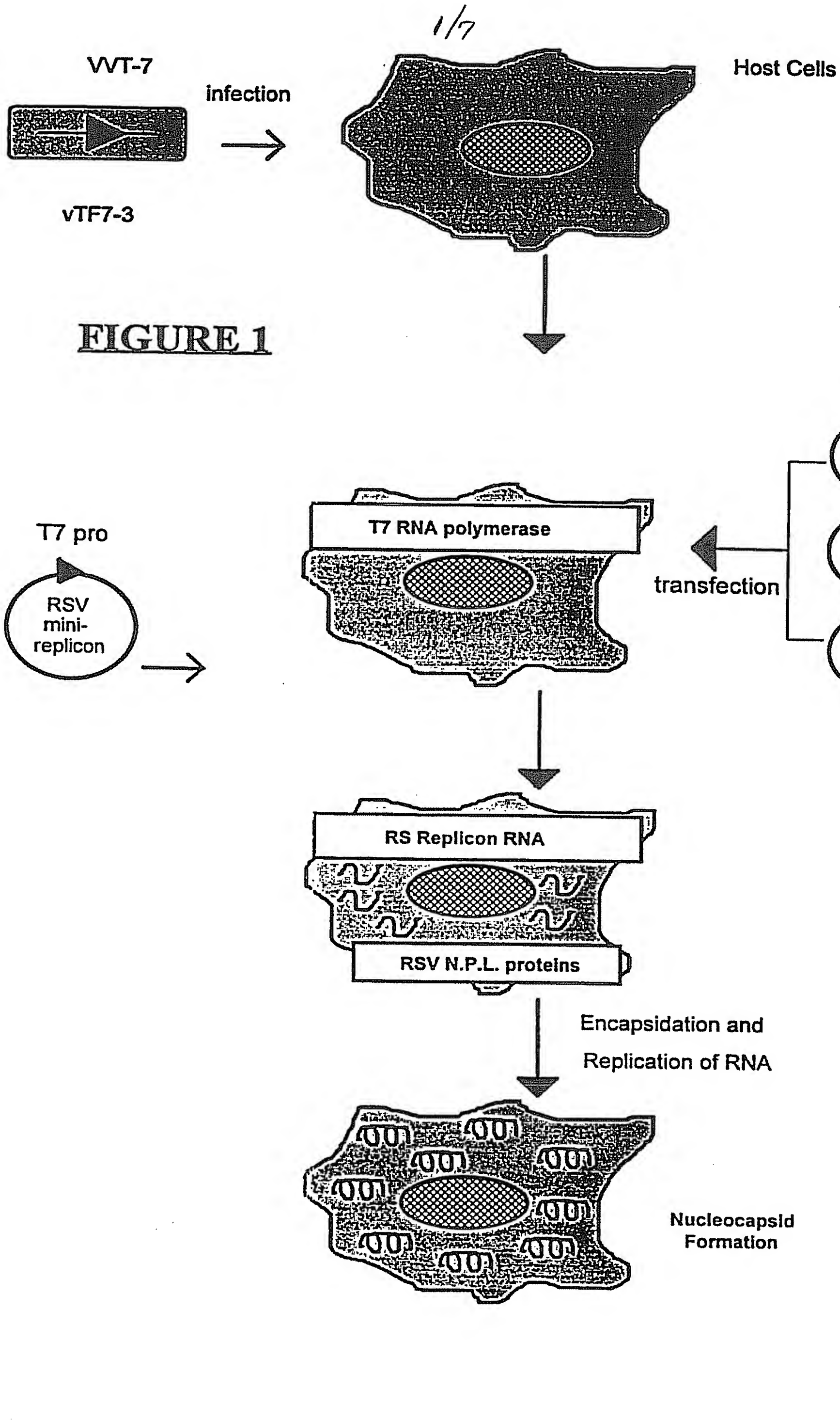
VVT-7
infection
vTF7-3
Host Cells
T7 pro
N
P
Pol
L
transfection
T7 RNA polymerase
T7 pro
RSV
mini-
replicon
RS Replicon RNA
RSV N.P.L. proteins
Encapsidation and
Replication of RNA
Nucleocapsid
Formation

FIGURE 1

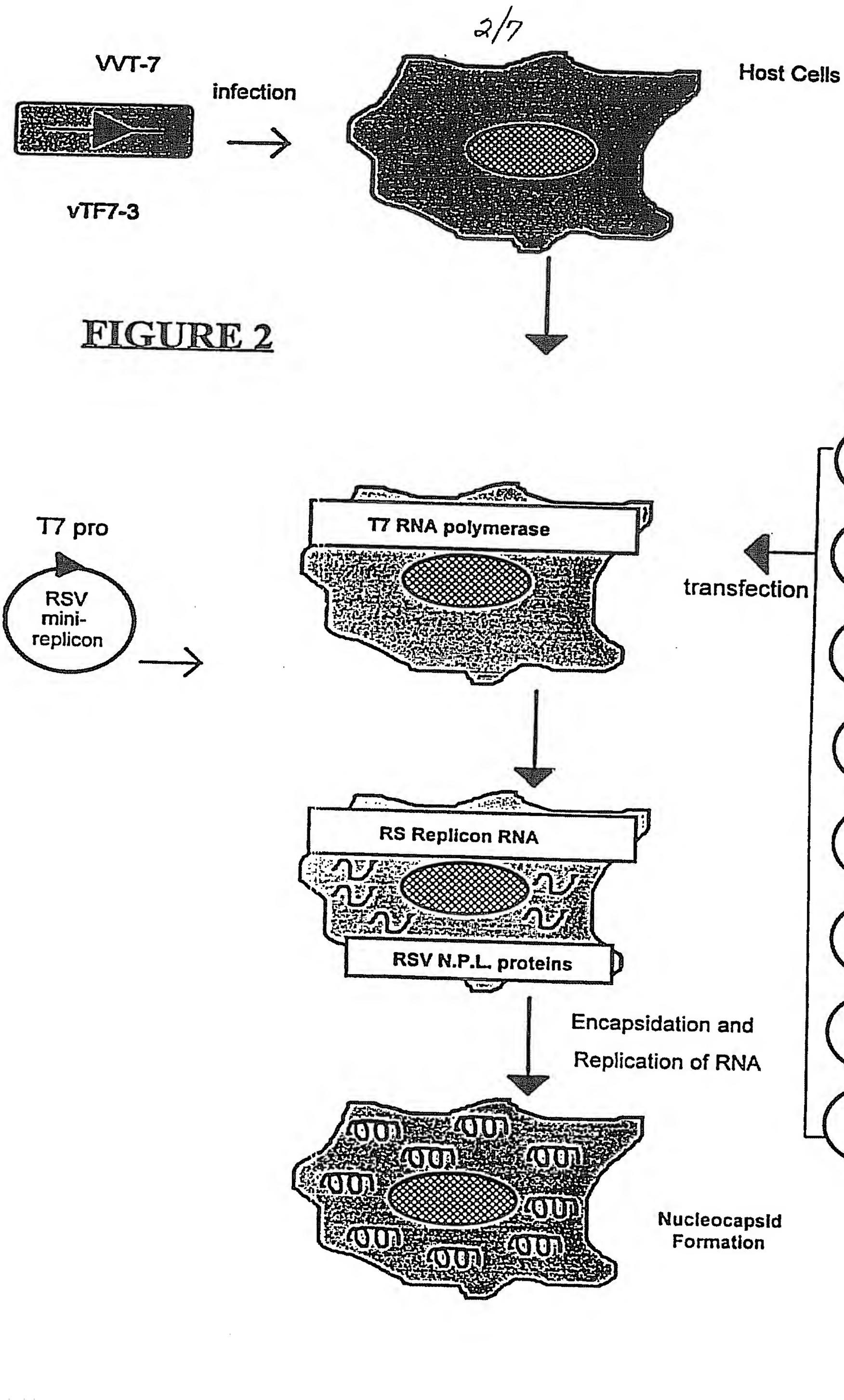
VVT-7
infection
vTF7-3
Host Cells
T7 pro
RSV
mini-
replicon
T7 RNA polymerase
RS Replicon RNA
RSV N.P.L. proteins
Encapsidation and
Replication of RNA
Nucleocapsid
Formation
T7 pro
transfection
N
P
Pol
L
M
SH
G
F
M2

FIGURE 2

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# FIGURE 3

RSV WILD TYPE ANALOGUE
(Mini Replicon)

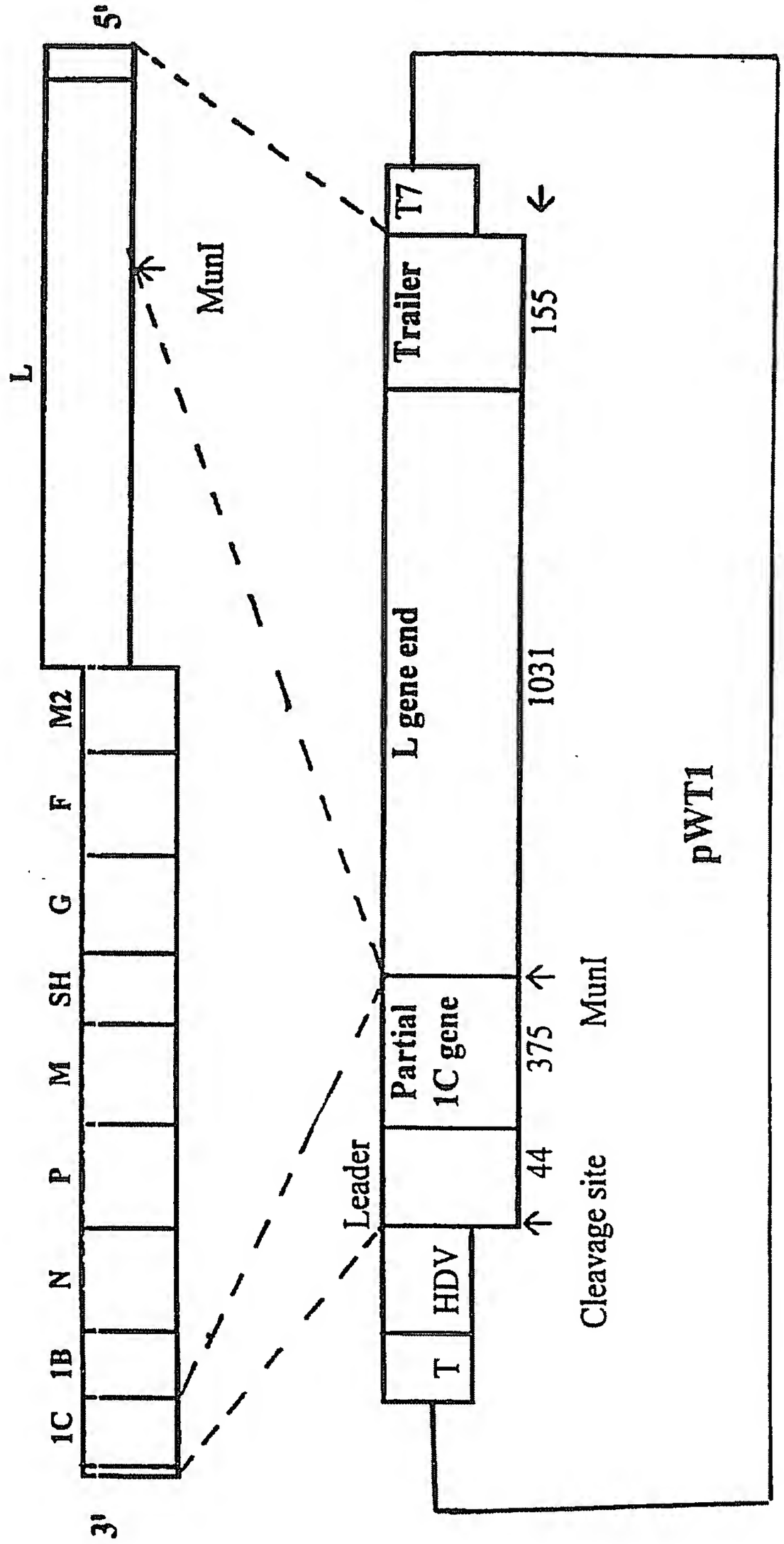
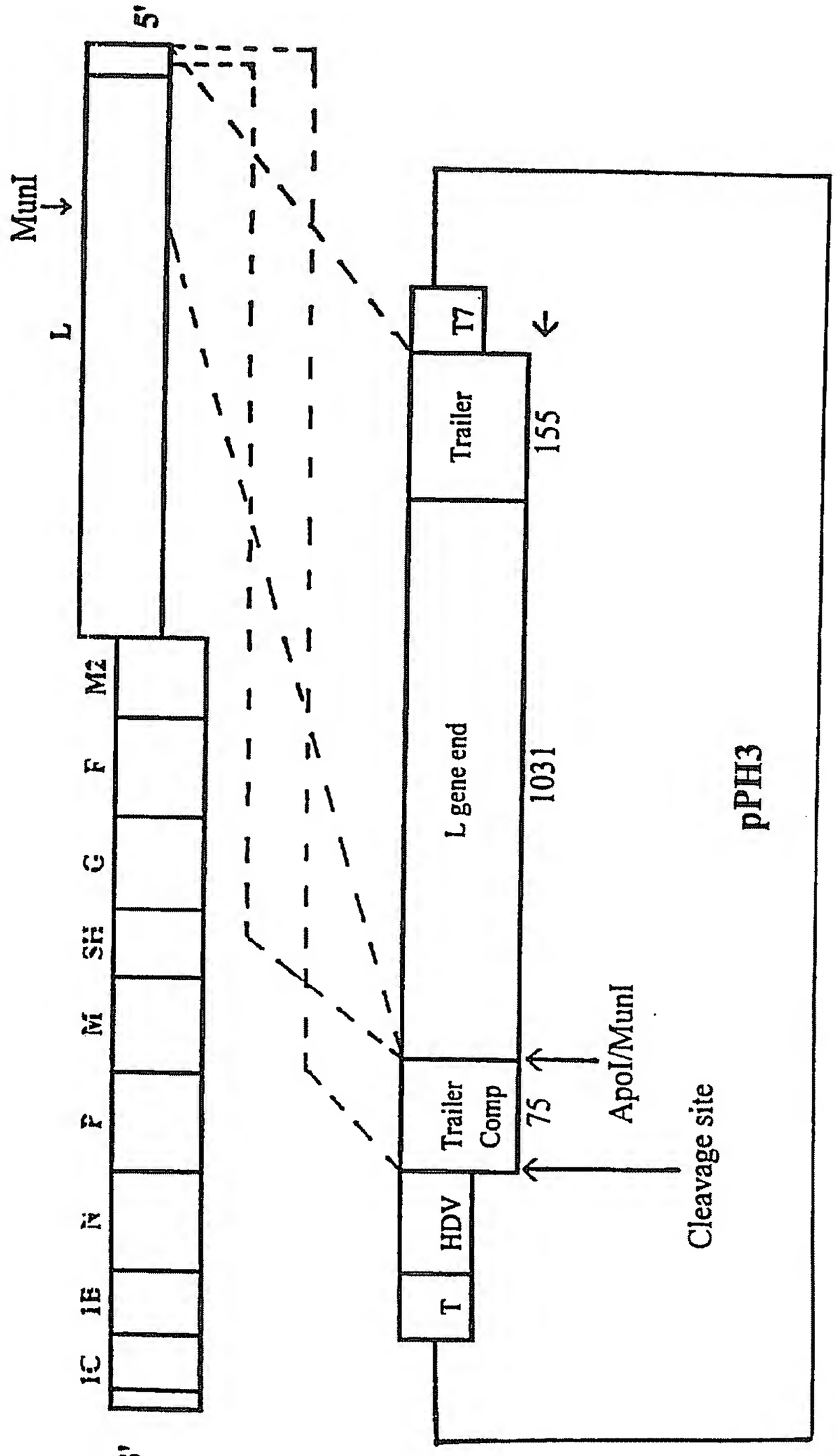


**FIGURE 4**

**RSV PANHANDLE ANALOGUE**
**(Mini Replicon)**



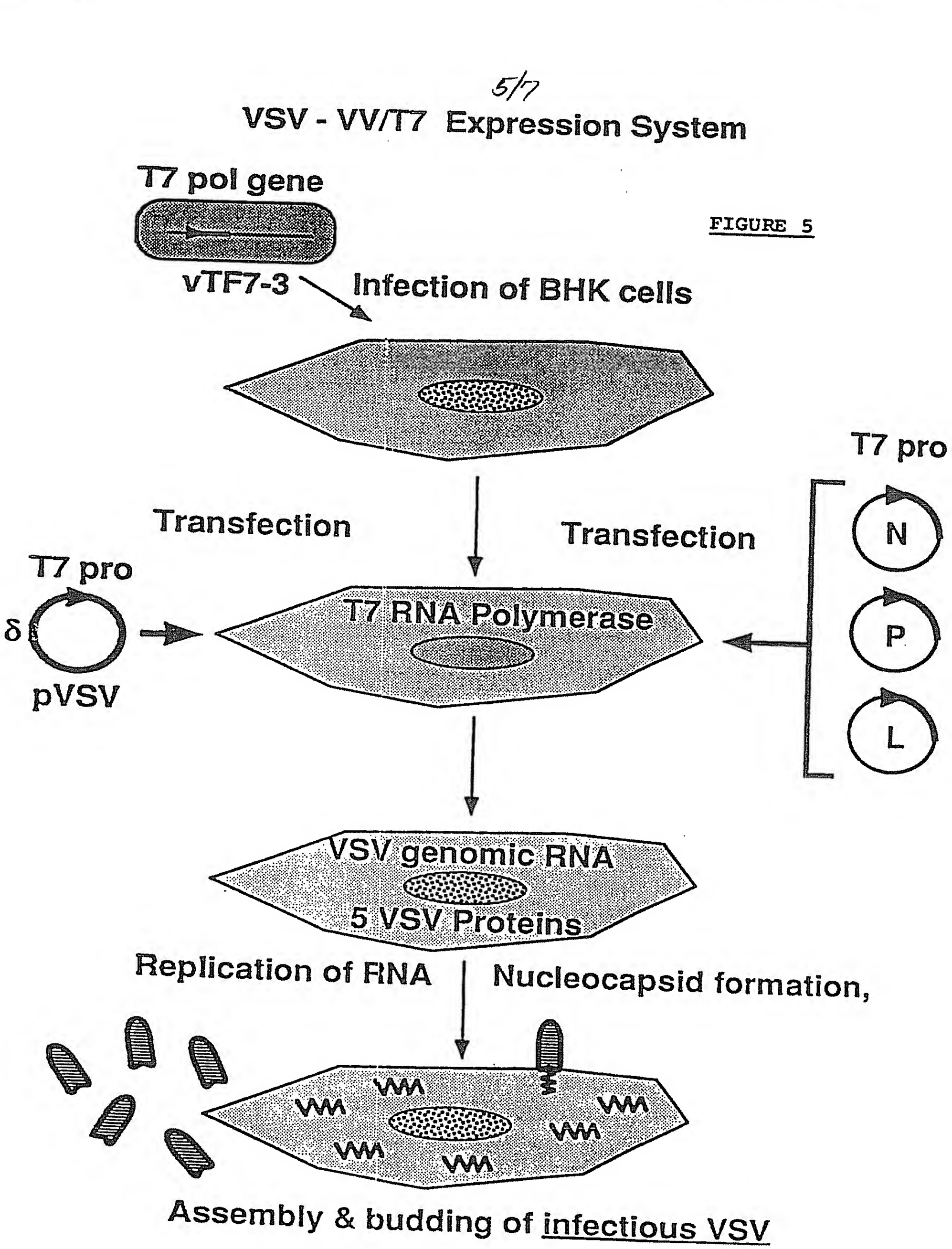

VSV - VV/T7 Expression System
T7 pol gene
vTF7-3
Infection of BHK cells
Transfection
Transfection
T7 pro
δ
pVSV
T7 RNA Polymerase
T7 pro
N
P
L
VSV genomic RNA
5 VSV Proteins
Replication of RNA
Nucleocapsid formation,
Assembly & budding of infectious VSV

FIGURE 5

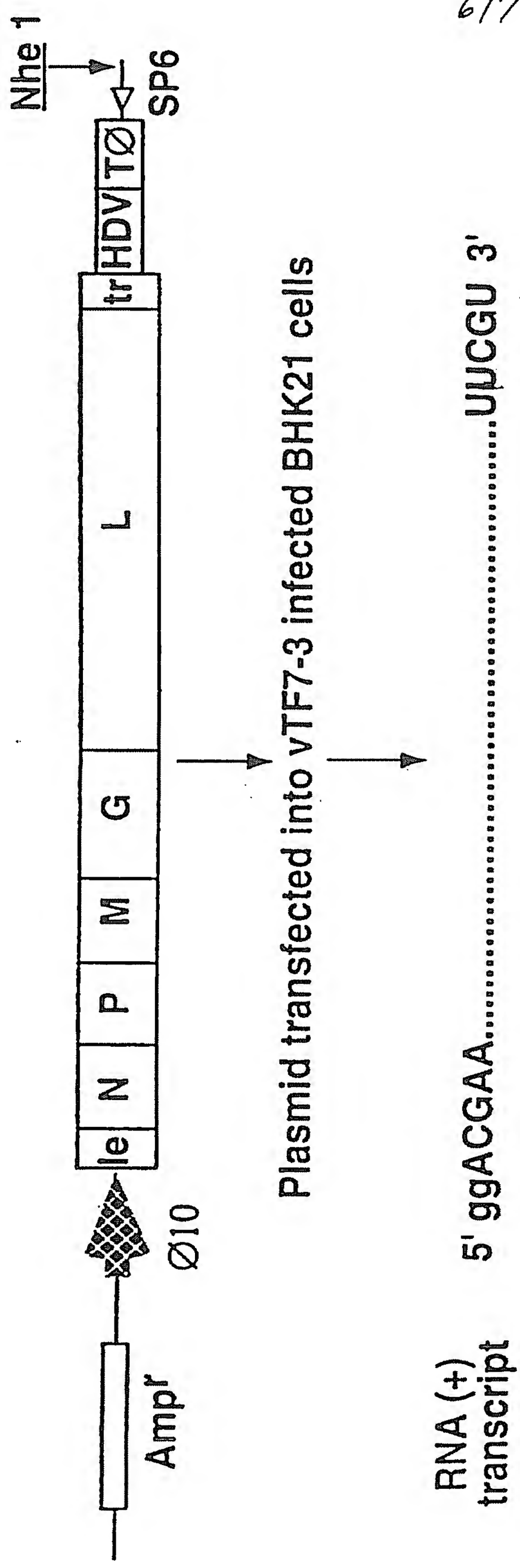

Nhe 1
SP6
tr
HDV
TØ
le
N
P
M
G
L
Ampr
Ø10
Plasmid transfected into vTF7-3 infected BHK21 cells
RNA (+) transcript
5' ggACGAA.....UUCG 3'

FIGURE 6

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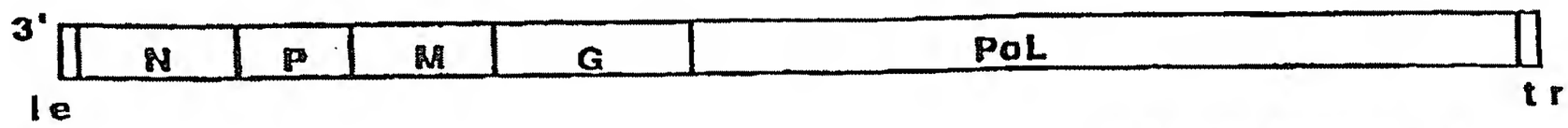
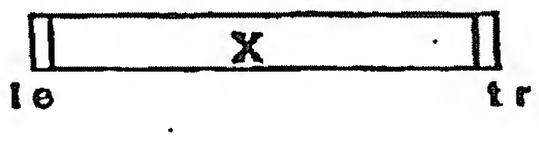
## VSV-Based Vectors

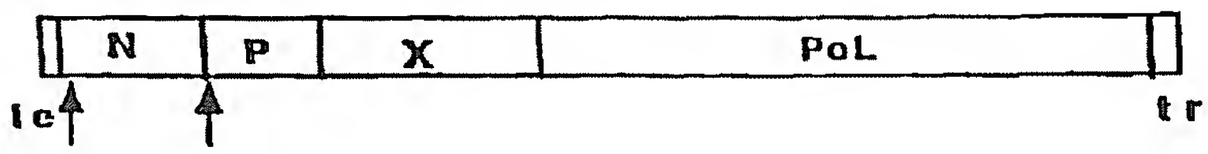



- N-nucleocapsid
- P-phosphoprotein
  M-Matrix
  G-glycoprotein
- L-Polymerase
- *-de novo synthesis required in replicatio

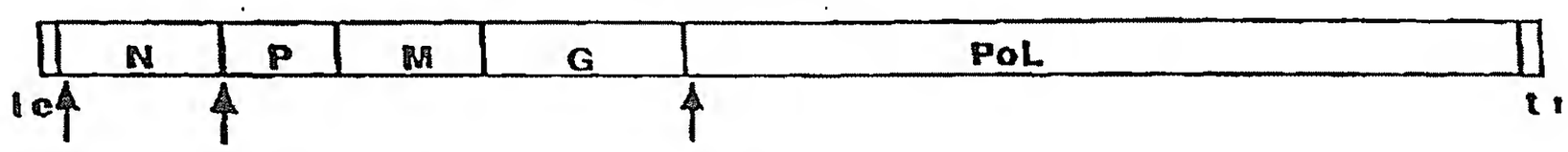
1. Transcribing




2. Transcribing & Replicating




3. Transcribing, replicating & spreading




X=Foreign gene

↑ Alternate position(s) at which foreign gene(s) may be inserted

FIGURE 7

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/12507

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : A61K 31/00, 39/00; C12N 15/00
US CL : 514/44; 435/320.1

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 435/320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAPLUS, EMBASE, BIOSIS, MEDLINE

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | US, A, 5,166,057 (PALESE ET AL) 24 NOVEMBER 1992, see entire document. | 1-42 |
| Y | Proceedings of the National Academy of Sciences USA, Vol. 88, issued February 1991, Pattnaik et al, "Cells that express all five proteins of vesicular stomatitis virus from cloned cDNAs support replication, assembly, and budding of defective interfering particles", pages 1379-1383, see entire document. | 1-8, 18-25, 35-42 |
| Y | VIRUS RESEARCH, Vol. 30, issued 1993, Wertz et al, "Workshop on 'Reverse genetics of negative stranded RNA viruses' Sponsored by the Juan March Institute, Madrid, Spain", pages 215-219, see entire document. | 1-8, 18-25, 35-42 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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INTERNATIONAL SEARCH REPORT

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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